DEVELOPMENT AND VALIDATION OF RP-HPLC AND HPTLC METHODS FOR THE SIMULTANEOUS ESTIMATION OF TENELIGLIPTIN HYDROBROMIDE HYDRATE AND GLIMEPIRIDE IN BULK AND TABLET DOSAGE FORM

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Submitted by

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CERTIFICATE

This is to certify that the dissertation work entitled **"DEVELOPMENT AND VALIDATION OF RP-HPLC AND HPTLC METHODS FOR THE SIMULTANEOUS ESTIMATION OF TENELIGLIPTIN HYDROBROMIDE HYDRATE AND GLIMEPIRIDE IN BULK AND TABLET DOSAGE FORM"** was carried out by **Ms. N. Abinaya (Reg. No. 261630751)**. The work mentioned in the dissertation was carried out at the Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore, Tamilnadu, under the guidance of **Mr. I. Ponnilavarasan, M.Pharm, (Ph.D),** for the partial fulfillment for the degree of Master of Pharmacy during the academic year 2017-2018 and is forwarded to the Tamilnadu Dr.M.G.R.Medical University, Chennai.

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This research work either in part or full does not constitute any of any thesis / dissertation.

Date: Signature

Place: Coimbatore **Mr. I. Ponnilavarasan, M.Pharm, (Ph.D)**

DECLARATION

I do here by declare that to the best of my knowledge and belief ,the dissertation work entitled **"DEVELOPMENT AND VALIDATION OF RP-HPLC AND HPTLC METHODS FOR THE SIMULTANEOUS ESTIMATION OF TENELIGLIPTIN HYDROBROMIDE HYDRATE AND GLIMEPIRIDE IN BULK AND TABLET DOSAGE FORM"** submitted to the Tamil Nadu Dr. M.G.R. Medical university , Chennai, in the partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmaceutical Analysis,** was carried out at Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore under the guidance of **Mr. I. Ponnilavarasan, M.Pharm, (Ph.D)** during the academic year 2017-2018.

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EVALUATION CERTIFICATE

This is to certify that the work embodied in the thesis entitled **"DEVELOPMENT AND VALIDATION OF RP-HPLC AND HPTLC METHODS FOR THE SIMULTANEOUS ESTIMATION OF TENELIGLIPTIN HYDROBROMIDE HYDRATE AND GLIMEPIRIDE IN BULK AND TABLET DOSAGE FORM"** submitted by **Ms**. **N. Abinaya (Reg. No:261630751)** to the Tamil Nadu Dr. M.G.R. Medical university, Chennai, in the partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmaceutical Analysis,** is a bonafide research work carried out by the candidate during the academic year 2017-2018 at KMCH College of Pharmacy, Coimbatore, Tamilnadu and the same was evaluated by us.

Examination Center: K.M.C.H College of Pharmacy, Coimbatore

Date:

Internal Examiner **External Examiner**

Convener of Examination

ABBREVIATIONS

HPTLC	High performance thin layer chromatography
HPLC	High Performance Liquid Chromatography
UV	Ultra violet
IP	Indian Pharmacopoeia
USP	United States Pharmacopoeia
M.W.	Molecular weight
BA	Bioavailability
e.g.	Example
i.e.	That is
%	Percentage
PDA	Photo Diode Array
mg	Milligram
ACN	Acetonitrile
MET	Methanol
ng	Nanogram
g	gram
µg	Microgram
ml	Milliliter
w/w	Weight by weight
v/v	Volume by volume
µg/ml	Microgram per milliliter
ng/spot	Nanogram per spot
RSD	Relative standard deviation
SD	Standard deviation
LOD	Limit of detection
LOQ	Limit of quantification
pH	Hydrogen ion concentration
°C	Degree Celsius
t	Time
min	Minute

h	Hours
R_t	Retention time
Abs.	Absorbance
Conc.	Concentration
Fig.	Figure
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
NMT	Not more than
HETP	Height equivalent to theoretical plate
ICH	International council for harmonization
API	Active pharmaceutical ingredients
TENE	Teneligliptin hydrobromide hydrate
GLI	Glimepiride

LIST OF TABLES

LIST OF FIGURES

CONTENTS

1. INTRODUCTION

Analytical method development and validation plays a main role in the discovery, development and manufacture of pharmaceuticals. Analytical methods are used to ensure identity, purity and potency of the drug products. There are various factors to be considered while developing analytical methods. At first collect the information about the analyte's physicochemical properties for e.g. pKa, log p, solubility etc.

The analytical chemistry is often be the described the area of chemistry responsible for characterizing the composition of the matter, both qualitatively (what is present) and quantitatively (how much is present). Analytical chemistry is not separate branch of chemistry, but simply application of chemical knowledge. The pharmaceutical analyst's in the respective quality control and quality assurance department (QA&QC) will check whether the products are complying with the standard or not. [1-2]

There are mainly two types of chemical analysis:

1. Qualitative (Identification)
2. Quantitative (Estimation)

1. Qualitative analysis: It is performed to establish composition of natural or synthetic substances. These tests are performed to indicate whether the substance or compound is present in the sample or not.

2. Quantitative analysis: This is mainly used to quantify any compound or substance in the sample.

Common techniques for analysis

1. Chemical methods [3]
    a) Volumetric or titrimetric methods
    b) Gravimetric methods
    c) Gasometrical analysis
2. Electrical methods
3. Instrumental methods
4. Biological and microbiological methods [4-7]

There are various types of instrumental methods available for the analysis of the drugs and drug products. Some of them are

- UV –Visible spectrophotometry
- Infra-red spectrophotometry (IR)

- X-ray spectroscopy
- Nuclear magnetic spectroscopy (NMR)
- High performance liquid chromatography (HPLC)
- High performance thin layer chromatography (HPTLC)
- Mass spectrometry
- Gas chromatography-mass spectrometry (GC-MS)
- Liquid chromatography-mass spectrometry (LC-MS)

1. Chemical methods:

a) Titrimetric and volumetric methods:

It involves the reaction of separation in substances to be determined with the appropriate reagent as the standard solution and volume of solution required to complete the reaction is determined.

b) Gravimetric methods:

In gravimetric analysis, a substance to be determined is converted to an insoluble precipitate in the purest form, which is then collected and weighed.

c) Gasometrical analysis:

Gasometry involve the measurement of volume of gas evolved or absorbed in a chemical reaction.

2. Electrical methods:

Electrical methods of involve the measurement of electric current, voltage or resistance in relation to the concentration of some spices in the solution. Electrical methods of analysis include

1. Potentiomety
2. Conductometry
3. Polarography
4. Voltammetry
5. Amperometry

3. Instrumental methods of analysis:

Instrumental methods involve the measurement of some physical properties of the compounds.

a) **Spectrophotometric methods:**

The spectroscopic method of analysis is depends upon the measurements of the amount of radient energy of particular wavelength emitted or absorbed by the sample.

b) **Chromatographic techniques:**

The chromatographic techniques are separation methods for the mixture of compounds and also applied for identification of compound of mixtures. Various chromatographic techniques are GC, HPLC, HPTLC, TLC, PC etc.

c) **X-ray methods:**

When high speed electrons collide with a solid target, X-rays are produced. From the remittent X-ray emission, it is possible to identify the certain emission peaks, which are characteristic of elements contained in target.

d) **Radioactivity:**

It involves measurement of the intensity of the radiation from naturally radioactive substances or induced radioactive substance arising from exposure of sample to a neutron source.

e) **Optical methods:**

i) Refractometer: based on measurement of refractive index of a liquids

ii) Optical rotation: for optically active compounds.

f) **Thermal methods:**

Changes in weight or changes in energy, record as a function of temperature like a Thermo gravimetry, Differential scanning calorimetry etc.

4. Biological and micro-biological methods:

Biological method is used when potency of a drug or its derivative cannot be properly determined any physical or chemical methods. They are called as bio-assays.

Micro-biological method is used to observe potency of anti-biotic or anti-microbiological agents. These methods include cup plate method and turbidimetric analysis.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY:

HPLC is one of the modern chromatographic techniques which are widely used in the fields of clinical research, bio-chemical research, industrial quality control etc. Application of these methods includes detection, analysis, determination, quantification and derivation of molecules from mixtures of biological, plant and medical samples. In column chromatography the solvent is just allowed drip through the column in high performance liquid chromatography, it is forced externally through the column at very high pressure up to 400 atmosphere. This will turn make the process lot faster. It also permits the very small particle size for the column packing material which offers a much greater surface area for interaction between the stationary phase and molecules flowing through it. High performance liquid chromatography be situated only of the best powerful tools now the analytical chemistry in means of the capacity to identify, separate then quantitate the compound that is existing in sample that is dissolved in every liquid.

HPLC method as the several advantages similar to rapidity, specificity, accuracy, precision, as well as ease automation now this method. HPLC method eliminates tedious extraction and isolation procedures.

The advancements in technologies trace concentration of compounds as low as parts per trillion can be easily too identified. HPLC can be applied to any sample such as, food, pharmaceutical, forensic sample, neutraceuticals, cosmetics, industrial chemical, environmental samples. There are two types of HPLC normal phase and reverse phase HPLC.

1) Normal phase HPLC:

The column is filled with tiny silica particles, and the solvent is non-polar-for example hexane. A typical column has an internal diameter of around 4.6mm, and length in the range of 150-250mm. polar compounds in the mixture which is passed through the column will stick longer to the silica longer to the polar silica than to the non-polar compounds. Hence the non-polar compounds will elute faster.

2) Reverse phase HPLC:

The column size is the similar as in normal phase HPLC, but silica is modified and completed non-polar attaching by long hydrocarbon chains to its surface with their 8 or 18 carbon atom in them. A polar solvent is used in this type of HPLC- for example, a mixture of an alcohol such as methanol and water is used. There will be a strong magnetism between the polar molecules and polar solvent in the mixture which is passing through the column. The attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution won't be as much. Therefore, polar molecules in the mixture will spend most of their time moving with the solvent. Because of van-der Waals forces the Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups present in the stationary phase. RP-HPLC method is the most commonly used for analysis. [8-13]

Characteristics features of HPLC:

HPLC method is having many advantages than the conventional method of analysis, in which the following is included;

- High resolving power and speed of separation.
- Accurate and quantitative measurement.
- Repetitive and reproducible analysis using the same column.
- Determination of several components in a single analysis.
- Easy collection of separated components for further characterization.
- A variety of solvents, column, and detectors are available.
- Readily automated data handling.

PRINCIPLE:

The principle of separation is based on the typical modes of separation of that of a classical chromatography

1) Adsorption
2) Partition
3) Ion exchange
4) Gel permeation

Adsorption chromatography

Adsorption is a surface phenomenon where the separations of components are achieved by adsorption to the stationary phase. It involves the accumulation of the liquid or gaseous solute on to the surface of the solid particle. The separation is based on affinity characters of the with stationary phase. Compounds having less affinity with stationary phase will eluted faster while those compounds more affinity with stationary phase will be eluted later.

Partition chromatography

In this method the stationary phase will be liquid coated with solid support. Here the separation is achieved based on the relative distribution solute between two liquid phases i.e; based on the partition co-efficient value. This type chromatography may be either normal phase or reverse phase depending on the nature of mobile phase and stationary phase.

Ion-exchange chromatography

This method involves reversible exchange of charged ions between the stationary ion exchange resin and liquid mobile phase. Separation is achieved due to the difference in strength of electrostatic interaction of solutes with the stationary phase.

Gel permeation chromatography

The mechanical sorting of molecules take place based on the size of the molecules in solution. Small molecules are able to permeate through the pores and therefore longer than larger molecules and hence molecules are elute first. [14-16]

Table 1: Various Types of HPLC

TYPE	SAMPLE POLARIZATION	MOLECULAR MASS RANGE	STATIONARY PHASE	MOBILE PHASE
Adsorption	non-polar to somewhat polar	100– 104	silica or alumina	non-polar to polar
Partition (reversed-phase)	non-polar to slightly polar	100– 104	non-polar liquid adsorbed or chemically bonded to the packing material	relatively polar
Partition (normal-phase)	To some polar to greatly polar	100– 104	highly polar liquid adsorbed or chemically bonded toward the packing material	relatively non-polar
Ion Exchange	extremely polar to ionic	100 – 104	ion-exchange resins complete of insoluble, high-molecular mass solids functionalized characteristically through sulfonic acid or amine groups	aqueous buffers by added organic solvents to moderate solvent strength
Size-Exclusion	non-polar to ionic	103 – 106	small, porous, silica or polymeric particles	polar to non-polar

Quantitation methods in HPLC:

The peak height and peak area measured by the detector signals. This measurement will give an account about the concentration or mass of the compound. For establishing this quantitation, some sort of calibration should be performed**.**

The major techniques for quantitation are:

1. Normalized peak area method
2. External standard method
3. Internal standard method
4. Method of standard addition method

Normalized peak area method

To determine the degradation product or impurity in a product, this method is used. The peak area of any one of the peak is referred to the normalized peak area. The response factor for each component is identified by this method.

External standard method

This method is done by injecting both standard and unknown sample. The unknown can be determined by calculating the response factor or calibration graph. The response factor R_f can be calculated from the following formula

$$R_f = \frac{standard\ peak\ area}{standard\ concentration}$$

There is no need of extensive sample preparation. The chromatographic conditions should be maintained constant during the separation of all standards and samples for better quantitation using external standard method. External standard methods are mainly used to ensure that the total chromatographic system is properly and can provide reliable results.

Internal standard method

A compound which is different from the analyte is used as the internal standard. But that compound should be resolved in the separation. The purpose of internal standard

is to mimic the nature and behavior of sample. For compounds which need pre-treatment or preparation this internal standard method is used.

The response factor is used for the determination of sample component in the original sample. Response factor is defined as the ratio of peak area of sample component (Ax) and the internal standard (AISTD). The formula is follow as,

$$Rf = \frac{A_x}{AI_{std}}$$

Based on the response factor and strength of internal standard (N_{ISTD}), the amount of analyte in the original sample can be calculated from the formula,

$$X = \frac{A_x}{R_F \bullet A_{ISTD}} \times N_{STD}$$

Method of standard addition

The method of standard addition can be used to provide calibration plot for the quantitative analysis. An important aspect of this method is that the response prior to spiking additional analytes should be high enough to provide to provide a reasonable S/N ratio (>10), otherwise the result will have poor precision. Mostly this method is used in the trace analysis. [17-18]

Instrumentation of HPLC:

The features of modern HPLC is illustrated in the block diagram comprise of components.

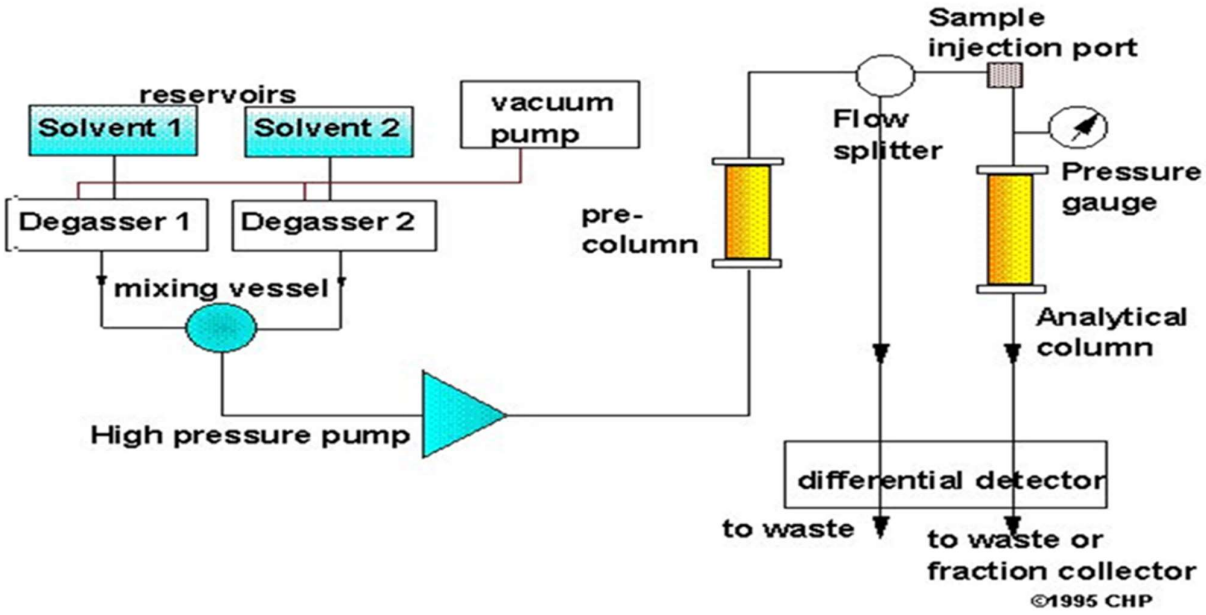

Fig.1: schematic diagram of HPLC

1. Pumping system:

HPLC pumping technique is required toward deliver metered amounts of mobile phase on a constant flow rate. Pumping system that is delivers solvent from one or more reservoirs be there available. Modern computer- or micro processer- controlled pumping systems are capable of delivering a mobile phase of either constant (isocratic elution) or either (gradient elution) composition, according to a defined programme.

Features of pumping systems:

High pressures up to 6000 psi, constant & reproducible flow, pulse free output, flow rate (0.1-10ml), adaptable to gradient flow, corrosion to resistant components. Easy to dismantle & repair and low maintenance cost.

2. **An injector:**

The sample solution is commonly introduced now toward the flowing mobile phase at or nearby the head of the column usage through means of an injection system based on injection value design which can operate at high pressure. Such as an injection

system has a fixed loop or a variable column devise which can operated manually or by an auto sampler. Partially filling of tuber may lead to poorer injection volume precision.

3. **Chromatographic column:**

A tube which contains the stationary phase. The stationary phase differentially interrelates through the sample constituent compounds they are carried along with the mobile phase HPLC column is packed with very fine particles (usually few microns in diameter).

Types of column:

- **Analytical column:**

  It performs the separation. It is a straight column with dimensions 4.0-8.0 mm (id) X 15cm or 25 cm length and particle size of 5or 10 micron. It contains 40000-60000 plates/m.

- **Preparative column:**
  It is 25-50mm (id) X 25cm (length) and used for preparative works. Its important parameters are sample load and transfer of analytical data to preparative data.

4. **Detector:**

The detector controls the sensitivity with which each compound can be detected and measured, once separated on the column. It must be capable of responding to concentration in all of the compounds of interest.

There are six main detectors used for HPLC. Refractive index (RI), ultraviolet (UV), fluorescence (FD), conductivity (CD), electrochemical (EC), and mass spectrometric (MS).

Ultraviolet visible (UV/Vis) absorption spectrometer is the most commonly used detectors for pharmaceutical analysis. [19-20]

Data system:

- For better accuracy and precision.
- Routine analysis: pre-programmed computing integrator.
- Data station/ computer needed for higher control levels.
- It adds automation options and complex data becomes more feasible.

System suitability parameters:

System suitability parameters toward ensue recognized intended for a specific process depend on the type of process which being validated. The simplest form of a system suitability test involves a comparison of the chromatogram trace with a standard trace. This allows the comparison of the peak shape, peak width, and base line resolution. These are few parameters that can be calculated to provide a quantitative system suitability test report.

- Number of theoretical plates (efficiency)
- HETP
- Capacity factor
- Peak asymmetry factor
- Resolution
- Tailing factor

i) Resolution (Rs):

The resolution Rs, two neighboring peaks is defined as the ratio of the distance between the two peaks maxims. This one of the located the variance among the retention times of double solutes divided in their average peak width. Intended for baseline separation, the ideal value of Rs is 1.5. It is calculated by using the formula,

$$R_s = \frac{Rt_2 - Rt_1}{0.5(W_1 + W_2)}$$

Where, Rt_1 and Rt_2 are the retention time of component 1 and 2 and

W□ and W□ are peak widths of component 1 and 2.

ii) Capacity factor (K):

Capacity factor, K, is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factors are measure of exactly how well the sample molecule is retained in a column or TLC plate during an isocratic separation. The ideal value of K ranges from 2-10 capacity factors can be determined by using the formula,

$$K = \frac{V_1 - V_0}{V_0} \times S$$

Where, V_1 = retention volume on the top of the peak (solute)

V□= void volume of the system

The values of K of individual band increase or decrease with changes in solvent strength. In reversed phase HPLC, solvent strength increases with the increase in the volume of organic phase in the water / organic mobile phase. Typically an increase in percentage of the organic phase by 10% by volume will decrease K bands by a factor of 2-3.

iii) Column efficiency (N):

Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of single peak. Smaller the band width, higher is the number of theoretical plates, indicating good column and system performance. Columns through N ranging from 2000 – 100,000 plates/meter are suitable intended for good system. Efficiency is calculated by means of using the formula,

$$N = 16\frac{Rt^2}{W^2}$$

Where, Rt is the retention time and W is the peak width.

iv) Peak asymmetry factor (As):

Peak asymmetry factor, As, can be used as a criterion for checking column performance. The peak half width, b, of a peak at 10% of the peak height, divided by the corresponding front half width, a, gives a asymmetry factor.

$$As = \frac{b}{a}$$

For a well packed column, an asymmetry factor of 0.9 to 1.1 should be achievable. [21-25]

Table 2: System suitability parameters and recommendations

Parameter	**Formula**	**Recommendation**
Theoretical Plates (N)	$N=5.54*(t_R/W_{0.5})^2$	In general should be > 2000
HETP(H)	H= L/N	HETP↓ Column efficiency↑
Peak asymmetry (As)	As= b/a	In general it should be 1
Tailing Factor (T)	T= (b+a)/2a	In general it should be 1
Resolution (R)	$R_s = \frac{2(t_2 - t_1)}{W_1 + W_2}$	R_s of > 2 between the peaks of interest.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY:

Thin layer chromatography (TLC) is also well-known as planar chromatography or flat chromatography similar all other chromatographic techniques, a multi stage distribution process. The most advanced form of TLC is commonly called high performance thin layer chromatography (HPTLC).

In most of the pharmacopoeial monographs thin layer chromatographic studies are the key identifying tests. The pharmacopoeial standard values are being used by industries as a basis for a quality control and current good manufacturing practices (CGMPs). In advancement of TLC be there high performance thin layer chromatography (HPTLC) remains robust, simple, rapid then an efficient tool in quantitative or qualitative analysis of compounds. HPTLC has an increased resolution than TLC which allows the clear and quantitative separation of the compounds. One of the enhancements is the use of higher quality TLC plates with finer particle sizes in the stationary phases. The separation can be further more improved by repeated development of a plate, using a multiple development device. As result, HPTLC offers better resolution and lower limit of detection (LODs).

HPTLC is the highest flexible, reliable then cost-efficient separation technique preferably suited for the analysis of botanical and herbal drugs etc. used through standardized then validated processes, it guarantees reproducible results. HPTLC is a vital element routine identification of complex finger prints of plant extracts and pharmaceutical products. It has established itself as the method of choice for handling complex analytical task involving identification of herbal drugs and botanicals.

High throughput analysis using HPTLC helps in the rapid analysis of large number of compounds. This field has been expedited by the requirements to provide analytical support of multiple drug targets emerging from the field of molecular biology, human genetics and functional genomics.

The power of thin layer chromatography has been improved by considering the chromatographic principles to enhance the speed and efficiency of separation by development of instruments to automate sample application, development of chromatogram and detection including accurate and precise quantification and identification. As the particle size of layer is decreases, the separation will be faster, more resolved and efficient. The particle size of the stationary phase has a narrower distribution range with an average size of 5µm, instead of average of 20µm for conventional TLC. The use of mechanical applicators like linomat 5, produce reproducible application reduces the diameter of the starting spots, compared to conventional TLC, only few volumes of sample are used in HPTLC, i.e; about one-tenth of the sample. The separation time is also reduced considerably.

Modern thin layer chromatography can be complementary to the HPLC technique. It allows the analysis of many samples in parallel, providing low cost analysis of simple mixtures for which the sample workload in high. The TLC plates acts as a "storage detectors" of the analytes if they are saved properly. [26-28]

Factors influencing the TLC / HPTLC separation and resolution of spots:

- Type of stationary phase
- Layer of thickness / binder in the layer
- Mobile phase (solvent system)

- Size and saturation of the developing chamber
- Sample volume to be spotted
- Solvent level in the chamber
- Relative humidity
- Temperature (R_f values with increase with rise in temperature)
- Separation distance

Features of HPTLC:

1. Simultaneous processing of sample and standard, it shows better analytical precision and accuracy, less need for internal standard,
2. Several analysts work simultaneously,
3. Lower analysis time and less cost per analysis,
4. Low maintenance cost,
5. Simple
6. Low mobile phase consumption per sample, [29-31]

HPTLC METHODOLOGY:

Set the analytical objective main that can exist quantification or qualitative identification or separation of twofold components/multicomponent combinations or optimization of analysis while before starting HPTLC. Process demands primary knowledge about the nature of the sample, namely, structure, polarity, volatility, stability and the solubility parameter of the sample.

Method improvement includes considerable test and error procedures. Selection of stationary phase exists quite easy. That remains to start with silica gel which is reasonable and nearly suits all kind of drugs.

Mobile phase optimization is done by using three level techniques.

i) First level involves use of neat solvents by considering the solubility of drugs and then by finding some such solvents can have average separation power for the desired drugs.

ii) Second level involves decreasing or increasing solvents strong point using hexane or water intended for respective purposes. This has change in their polarity.

iii) Third level involves annoying of mixtures instead of neat particular solvents since the selected solvents first and second level which can further be optimized through the use of modifiers like acids or bases are desired volumes.

Analytes are detected using either fluorescence or absorbance mode. Optimization can be started only afterward a reasonable chromatogram which can be finished by slight change in mobile phase composition.

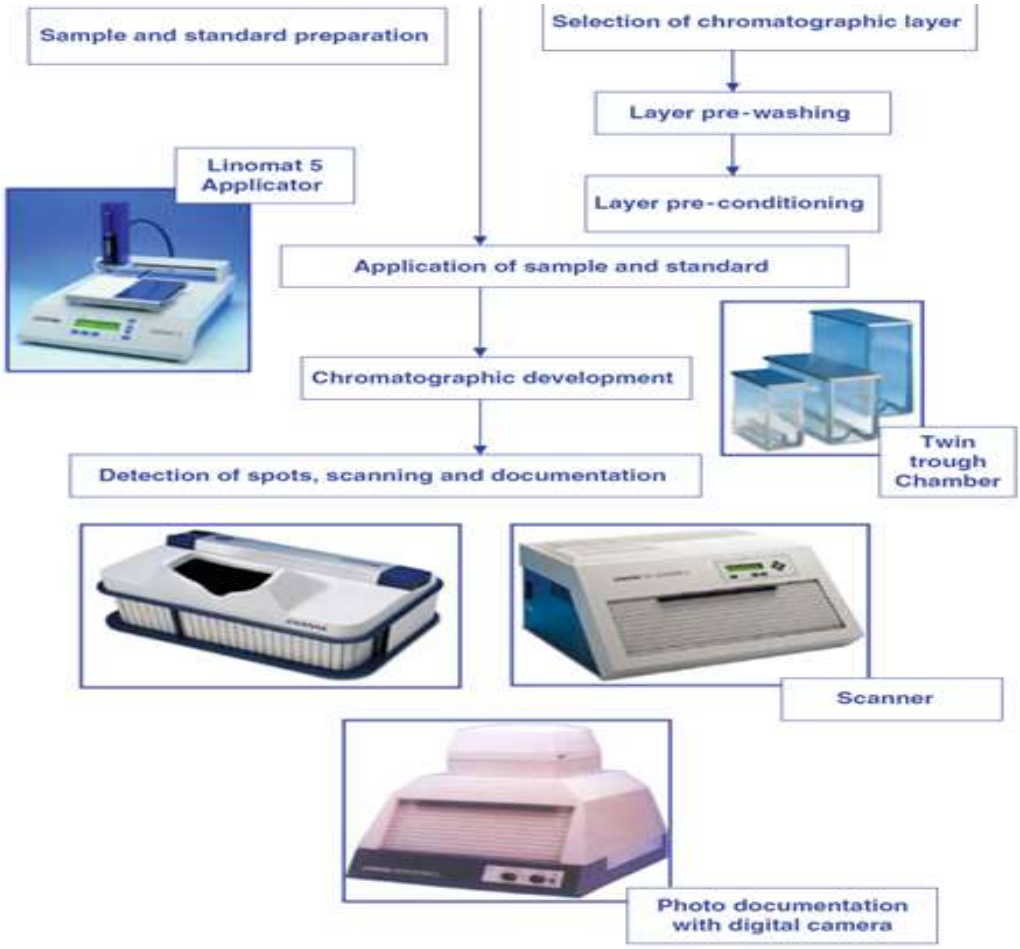

Fig. 2: steps involved in HPTLC

1. Sample preparation:

Normal phase chromatography: non polar solvents

Reverse phase chromatography: polar solve

2. Selection of chromatographic layer:

Precoated plates of different support material (different solvents) are available for 80% of analysis. Generally plates of 20 × 20cm or 10 × 10cm size having 100-250mm adsorbent thickness are used.

- Alkaloids and steroids – aluminium oxide, silica gel 60 GF□□□
- Amino acids, dipeptides, sugars and alkaloids – cellulose
- Non-polar substances, fatty acids, carotenoids and cholesterol – RP-2 and RP-8
- Preservatives, barbiturates, analgesics, phenothiazine – hybrid plates (RP-WF□□□)

3. Layer pre washing:

It is a purification step to remove water vapours present in the plate. This step also removes the volatile impurities which might get trapped in the plates. Mainly methanol is as a solvent for washing by ascending or descending technique. This step is very essential for quantitative evaluation and stability testing of drugs.

4. Conditioning of pre coated plates:

Freshly open box of plates do not require activation. Plates exposed to high humidity or kept on hand for long time is to be activated. Activation is done by using the plate in oven at 110 to 120ºc for 30minutes prior to spotting. Dried plates are stored at dust free atmosphere.

5. Sample application:

The samples are applied on to the separation layer, either as spots through capillary tube or as narrow bands using the spray-on technique by the help of a sample applicator.

The criteria like precision of applied volume, small size of application zone and exact positioning of sample are essential for the quality or repeatability of the analysis. Usually the concentration range between 0.1-1µg/µl. Above this causes poor separation. Band wise sample request open through linomat 5 applicator or automatic TLC sampler 4 which operates the best separation regarding sample and application volumes. Nitrogen gas, spray the sample and standard from syringe on TLC plates as bands. Band wise application provides better separation which gives high response to densitometer giving good results. By using spray-on technique the applied volume can be easily adjusted to match the required detection limit of the analytical task.

6. Selection of mobile phase:

Trial and error or one's own experience and literature helps in the selection of mobile phase 3-4 component mobile phase should be avoided. Multi component mobile phase while used not mentioned for extra use and solvent composition exists expressed by volume (v/v) and sum of volumes is usually 100 twin through chambers are used which requires only 10-15ml of mobile phase. Components of mobile phase must be mixed and introduced now to the twin through chamber.

7. Pre- conditioning (chamber saturation):

Un-saturated chamber causes high RF values and improper solvent front. Saturated chamber through inside layer by filter paper for 30 minutes later to development uniform distribution of solvent vapours-less solvent meant for the sample to travel lower RF values.

8. Chromatogram development:

Chromatogram is developed by capillary force. The developing solvent (mobile phase) migrates through the layer (stationary phase) over a defined distance called solvent front. During this process the sample is separated into fractions/bands (components). After evaporation of the mobile phase by keeping the layer/plate in hot air oven or at room temperature, all fractions remain stored on the layer.

9. Derivatization:

It is a special advantage of planar chromatography that fractions are stored on the plate and can be derivatized after chromatography. By Derivatization, substances do not respond to visible or UV light can be rendered detectable. In case of certain compounds, substances or classes of substances can be identified by using specific reagents.

Eg: Ninhydrin, Dragandroff's reagent

10. Chromatogram evaluation:

The chromatogram is evaluated under UV light or white light detection under UV is first choice nondestructive. The various methods involve visual inspection, electronic image processing, video densitometry and documentation to quantitative determination by means of monochromatic light in a classical densitometer. This additionally facilitates measurement of spectral information. Two types of ultra violet light are required by inspecting thin layer chromatogram.

- **Long wave UV light 366nm:**

Some substances may naturally inherent or reagent may induce fluorescence which appears at bright spots, often differently coloured, on a back ground. The sensitivity of this detection method is proportional with the intensity of the long-wave UV light and also as more visible light is eliminated. A fluorescent compound F254 contained in the layer does not interfere with this detection method.

- **Short wave UV light 254nm:**

Substance absorbing at this wavelength become visible provided that TLC layer contains a fluorescent indicator F254. This substance appear as dark spots on a back ground UV intensity and complete elimination visible light are less critical for this detection method.

Spots of non-fluorescent compound ethambutol, dicylomine etc are dipping the plates in 0.1% iodine solution.

11. Scanning and documentation:

A standard flatbed scanner is a viable tool for quantitative thin layer chromatographic (TLC) plate analysis. The imaging scanner uses a gas filled proportional counter, which can entire TLC lane in less than one minute. The system includes winCATS software for instrumental control, data analysis, and report generation. The scanner converts bands into peaks. Peak height and area are related to the concentration of substance in the spot. Quantification of peak is automatically performed, and a report showing the method used, chromatogram, and percent of total activity for each peak is provided. [32-37]

Parameters that are affected by the changes in chromatographic conditions are:

- Retention factor (R_f)
- Peak purity

1. Retention factor (R_f):

Retention factor is defined such as the quantity of separation due to the solvent migration through the sorbent layer as shown in the formula. It depends on time of development and velocity coefficient or solvent front velocity.

$$R_f = \frac{\text{Migration distance of substance from origin}}{\text{Migration distance of solvent front from origin}}$$

2. **Peak purity:**

The null hypothesis "these spectra are identical" can in this case (purity) with two sided significance. Through the purity test the spectrum in use at the first peak slope is correlated by the spectrum of peak maximum [r (s, m)] then the correlation of spectra taken at the peak maximum by the one from the down slope or peak end [r (m, e)] which is used by way of a reference spectra for statistical calculation. An error probability of 1% only is rejected if the test value is greater than or equal to 2.57. [38]

Quantitative analysis:

The primary goal of the method is to provide validated methods to be used for the quantification of the compounds most correlated with pharmacological activity or qualitative markers as determined by the primary pharmacological literature, product labeling and survey of experts. The method will be selected from the primary analytical literature by a methods selection committee with priority given to compendia methods when available. In this context, validation consists minimally of a two lab validation using the same procedures, samples and reference standards. Primary factors for considering methods as appropriate include accuracy of the findings, speed, basic ruggedness, applicability to a large segment of the manufacturing community, and

avoidance of the use of toxic reagents and solvents. In an attempt to promote harmonization, primary consideration is given to those methods which are already accepted in official pharmacopeias. When necessary, comparative tests shall be conducted to determine which of the available method is most appropriate. [39]

VALIDATION OF ANALYTICAL PROCEDURE:

Significance of method validation:

The quality of analytical data is a key factor in the success of a drug development programmer. The process of method development then validation takes a complete impact on the quality of these data.

- To trust the method
- Regulatory requirement

Different types of validation characteristics:

- Precision
  - ✓ Repeatability
  - ✓ Inter and intraday precision
- Accuracy
- Specificity/selectivity
- Linearity range
- Limit of detection (LOD)
- Limit of quantification (LOQ)
- Robustness
- Ruggedness
- System suitability

Generalized validation process for HPLC assay methods

Validation is the process of collecting documented evidence that the method performs according to its intended purpose. The validation process as follows:

1. **Precision:**

The closeness of contract between a sequence of measurements of multiple samplings of the same homogeneous sample under prescribed condition. The precision

of a test method is usually expressed as the deviation or relative deviation of a series of measurements.

Precision may be considered at three levels:

- Repeatability
- Interday and
- Intraday Precision.

Acceptance Criteria:

- Percentage Relative deviation (%RSD) NMT 1 % (Instrument precision)
- (%RSD) NMT -2% (Intra- assay precision)

2. Accuracy:

The ICH guideline mentions that accuracy requirement be determined by a smallest of nine determinations over a minimum of three concentration levels covering the identified range. The spiked samples are prepared now triplicate at three levels over a range that covers 80 -120% of the target concentration for assay methods .There are several methods that can be used for determining accuracy.

In this case, method accuracy is the agreement between the differences in the measured analyte concentration and the known amount of analyte added. That is the accuracy or % recovered is calculated as:

$$\text{Accuracy} = \frac{C_m \times 100}{C_t}$$

Where; C_m is the measured concentration

C_t is the theoretical concentration.

Accuracy has also been reported as; when a sample is analyzed the measured value should ideally be identical to the true value. Accuracy is represented and determined by the recovery experiments. The usual range is being 10% above or below the expected range of claim. The % recovery was calculated using the formula,

$$\%\,\text{Recovery} = \frac{(a+b)-a}{bX100}$$

Where,

a – Amount of drug present in sample

b – Amount of drug added to the sample

Acceptance Criteria:

- For an assay method, mean recovery will be 100%± 2% at each concentration over the range of 80-120% of the target concentration.

3. Limit of Detection:

The condition is previous amount of analyte now a sample analyzed that contain exist detected but not necessarily be quantitated below the stated experimental conditions.

Following are different approaches:

i. Based on Signal to Noise Ratio Method:

The LOD can be expressed as a concentration at specified signal-to-noise ratio obtained from samples spiked with analyte. A signal-to-noise ratio is 3:1 is generally considered to be acceptable.

ii. Based on the Deviation of the Response of the Slope:

- Prepare the blank solution as per test method inject six times into the chromatographic system.
- Similarly prepare the linearity solution starting from lowest possible concentration of analyte to 150% of target concentration and establish the linearity curve.

The detection limit (DL) may be expressed as:

$$LOD = 3.3\ \sigma / S$$

The slope shall be estimated from the calibration curve of the analyte.

4. Limit of quantification:

It is lowest amount of analyte in a sample, which can be quantitatively determined is LOQ.

$$LOQ = 10\ \sigma / S$$

Acceptance Criteria:

- In Pharmaceutical application, the LOQ is typically set at minimum 0.05% for active pharmaceutical ingredients.

5. Linearity range:

The linearity of an analytical method is its ability to evoke test results that are consider to the analyte concentration in samples within a given range. The working sample concentration tested for accuracy should be in the linear range. The demand that the method is linear is to be justified with additional mention of zero intercept by processing data by linear least square regression (R^2).

$$y = mx + C$$

For the method to be linear the R^2 value should be near to1. Where **y** is the measured output signal, **x** is the concentration of sample, **m** is the slope, C is the intercept.

The range of an analytical method is the interval between the upper and lower levels of the analyte (including these levels) that have been demonstrated to be determined with precision, accuracy linearity using the method as written.

Acceptance criteria:

Coefficient of correlation should be NLT 0.99.

6. Robustness:

That one is a measure of the method's capability to remains unaffected done slight then deliberate deviations now the parameters, which provides an indication of his reliability throughout normal usage.

For example in a chromatographic method, the typical method parameters need to change deliberately verify during method validation:

Flow rate : (+/- 0.2ml/minutes).

Mobile phase composition : (+/- 10% of organic phase).

PH of buffer in mobile phase : (+/- 0.2 units).

For Variations:

1. System suitability should meet the acceptance criteria as per test method.
2. If system suitability doesn't meet, narrow the variation range carryout the experiment again to meet system suitability.

7. **Ruggedness:**

Ruggedness is near a measure of reproducibility of the test effects under normal operational conditions though laboratory to laboratory and after analyst to analyst.

The following are the typical method parameters needed to be tested during the method validation:

- Analyst-to-Analyst variability.
- Column-to-Column variability
- Different days [40-57]

Table 3: Method Validation Requirements (ICH)

METHOD VALIDATION REQUIREMENTS	ACCEPTANCE CRITERIA
Precision Assay repeatability Intermediate precision (Ruggedness)	≤ 1% RSD ≤ 2% RSD
Accuracy Mean recovery per concentration	100.0% ± 2.0%
Limit of detection Signal to-to-noise ratio	≥ 3:1
Limit of quantification Signal to-to-noise ratio	≥ 10:1
Linearity/Range Correlation coefficient y-Intercept Visual	>0.99 ± 10% Linear
Robustness System suitability met Solution stability	 yes ± 2% change from time zero

2. REVIEW OF LITERATURE

Ganesh Kumar[58] **et al.** Developed and validated simple and new stability indicating RP-HPLC method for the identification of teneligliptin and its degradants on kromasil 100-5C18 (250×4.6mm, 5µm) column using pH 6.0 phosphate buffer and acetonitrile (60:40 v/v) as mobile phase in isocratic mode of elution of flow rate of 1.0ml/min. The column effluents were monitored by a variable wavelength UV detector at 246nm. The method was validated as per ICH guidelines. Forced degradation studies of teneligliptin were carried out under acidic, basic, neutral (peroxide), photo and thermal conditions for 48hrs at room temperature respectively.

Kadam V.N. [59] **et al.** Proposed simple accurate, precise and reproducible RP-HPLC method was developed for the determination of Voglibose (VGB), Glimepiride (GLM) and Metformin HCL (MET). RP-HPLC method was developed on jasco 2075 HPLC systems with fine pack ODS C18 column (250mm) and using a mobile phase mixture containing mixed acetonitrile: phosphate buffer in the ratio of 85:15 (pH 4). The flow rate was 1ml/min and effluent was monitored at 223nm. The retention time of voglibose, glimepiride and metformin HCL were 2.3, 3.8 and 5.1 min respectively. The method was validated in terms of linearity, precision, accuracy, specificity and system suitability parameters. The proposed method results were found to be satisfactory and are suitable for simultaneous estimation of voglibose, metformin and glimepiride for routine quality control drugs.

Sunil R. Dhaneswar [60] **et al**. validated simple, precise and accurate HPTLC method for simultaneous estimation of metformin hydrochloride (MET), atorvastatin (ATV) and glimepiride (GLM) as the bulk drug and in tablet dosage forms. Chromatographic separation of the drug was performed on aluminium plates precoated with silica gel 60F□□□ as the stationary phase and the solvent system consisted of water: methanol: ammonium sulphate (1: 1: 4 v/v/v). Densitometric evaluation of the separated zones was performed at 237nm. The three drugs were satisfactorily resolved with Rf value as the 0.37±0.02 and 0.59±0.02, 0.75±0.02 for MET, AVT, GLM respectively.

Gopal S. Irache [61] **et al**. developed and validate a simple, rapid and reproducible gradient high performance reverse phase liquid chromatographic method for the estimation of teneligliptin and metformin in bulk drug sample and pharmaceutical dosage forms using cromosil (C18, 250×4.6mm, 5µm) column with mobile phase composition of methanol and water (pH 3.5) 50:50 v/v. Flow rate of 0.7ml/min and UV detection at 242nm was maintain during the entire study. The retention time of metformin and teneligliptin was found to be 2.45 min and 6.21 min respectively. Linearity was observed over concentration range of 2-10µg/ml and 50-250µg/nl for teneligliptin and metformin respectively. The accuracy of the proposed method was determined by recovery studies and found to be 98-101%. The proposed method was validated and results conformed to ICH parameters.

Abdul Bari Mohd [62] **et al.** A simple, rapid sensitive method was carried out on a 5µm particle octadesyl silane (ODS) column (250×4.0 mm) with acetonitrile 0.2M phosphate buffer (pH 7.4) 40;60 v/v as a mobile phase at a flow rate 1ml/min and quantification was achieved at 228nm using PDA detector. The correlation coefficient (r^2) was found to be 0.999 over the concentration range of 0.2 to 2 µg/ml for glimepiride. The method was validated for linearity, accuracy and precision. The limit of detection and limit of quantification were found to be 0.38 and 1.17 µg/ml respectively.

Vishnu C. Shinde [63] **et al.** developed and validate simple, rapid sensitive and specific UV spectrophotometric and high-performance thin layer chromatographic (HPTLC) methods for the determination of Teneligliptin Hydrobromide both in bulk drug and pharmaceutical dosage form were developed and validated. In UV spectrophotometric method, the solutions of Teneligliptin HBr were prepared in water. The standard solution of Teneligliptin HBr showed maximum absorption at wavelength 243.5 nm. The drug obeyed Beer-lamberts law in the concentration range of 10-90 µg/ml with coefficient of correlation (r^2) of 0.999. For HPTLC method, the method employed aluminium plates precoated with silica gel G60 F□□□ as the stationary phase. The solvent system consisted of toluene: chloroform: ethanol: diethyl amine in the proportion of 4: 4: 1: 1 v/v/v/v. This solvent system was found to give compact spots for Teneligliptin HBr with Rf value 0.16±0.01. Densitometric analysis of Teneligliptin HBr was carried out in the absorbance mode at 254nm. Linear regression analysis showed good linearity (r^2=0.998)

with respect to peak area in the concentration range of 100-600 ng/spot. The developed methods were validated as per ICH guidelines.

Deepak Patil [64] **et al.** accurate, precise and reproducible high performance liquid chromatographic method was developed for quantitative estimation of metformin and teneligliptin simultaneously in tablet dosage forms. Younglin (S.K.) gradient system UV detector and C (Agilent) column with 250mm×4.6mm i.d. and 5µm particle size. Methanol: water 0.05% OPA (50:50) was used as the mobile phase for the method. The detection wavelength was 235nm and flow rate was 0.7ml/min. In the developed method, the retention time of metformin and teneligliptin was found to be 2.1 min and 7.6min. The developed method was validated according to ICH guidelines. The linearity, precision, range, robustness was within the limits as specified by the ICH guidelines. The proposed method can be used for the routine quality control analysis of metformin and teneligliptin respectively.

Pradnya N. [65] **et al.** a simple reverse phase high performance liquid chromatography method was developed and validated for simultaneous determination of metformin hydrochloride (MET) and glimepiride (GLM) in combination and estimation of their principal degradation products. The separation was achieved using JASCO finepak SIL (250mm×4.6mm i.d. 5µm) at ambient temperature. The optimized mobile phase composed of an aqueous phase (20 mM phosphate buffer, adjusted to pH 3.0) and an organic phase (methanol: acetonitrile, 62.5: 37.5) in the ratio of 80:20. The flow rate was 1ml/min, and the analytes were detected at 230nm. The developed method was validated for accuracy, precision, specificity, linearity and sensitivity. The chromatographic analysis time was accurately six minutes with the complete resolution of MET (Rt 2.75minutes) and GLI (Rt 5.87minutes). The method exhibited good linearity over the range of 5-30 µg/ml for MET and 1-10 µg/ml for GLI. The drugs in combination were subjected to various stress degradation studies as per the ICH guidelines.

Mastanamma Shaik. [66] **et al.** developed and validate simple, sensitive, linear, precise and accurate method by reverse phase high performance liquid chromatography for the simultaneous estimation of metformin (MET), losartan(LOS) and glimepiride (GLM) in bulk and their combined tablet dosage form. The separation of these drugs was based on

the use of luna C18 (250×4.6mm, i.e. 5µm) column in a gradient mode. Mobile phase consisted of methanol (solvent A) and 0.1% orthophosphoric acid (OPA, solvent B) was set with gradient programming for 18min and was delivered at 1ml/min flow rate and effluents are achieved with variable wavelength photodiode array detector at 284nm. The retention time of MET, LOS and GLI were found to be 3.11, 7.12 and 13.52mins respectively. The percentage assay of MET, LOS and GLI was found to be 100.5%, 100.5% and 100.4% respectively. Calibration curve were linear for MET, LOS and GLI at concentration ranges of 30-450 ng/ml and 15-225 ng/ml and 1-18 ng/ml with the regression co-efficient of 0.999 for all three drugs and precise with (%RSD>2). The drug was subjected to various stress condition of acid and base hydrolysis, oxidation, photolysis, thermal degradation and condition.

Vedantika Das [67] **et al.** developed and validate simple, accurate, precise and economical HPLC method has been developed and validated for the estimation of teneligliptin hydrobromide hydrate (THH) in bulk and tablet dosage form. Separation was achieved on a prontosil C8 column using a mobile phase consisting of acetonitrile: dihydrogen potassium phosphate buffer in 60: 40 (v/v) adjusted with o-phosphoric acid pH 3.0. Gradient elution at a flow rate of 1ml/min and UV detection at 246nm. Linearity was observed in the concentration range of 30-150 µg/ml. The retention time of teneligliptin was 2.47min. The proposed methods were validated according to the ICH guidelines.

M. Vijaya Kumari [68] **et al.** developed and validate simple, rapid, precise and accurate gradient reverse phase HPLC method was developed and validated for the determination of teneligliptin in commercial tablets. Separation was achieved on a C8, 150×4.6mm i.d., 5µm stainless steel analytical column i.e. using a mobile phase a consisting of 1ml OPA in 1000ml water and acetonitrile: methanol (80:20) v/v as mobile phase B with pH 5.3 at a flow rate of 1ml/min and UV detection at 213nm. The retention time was found to be 9.8min. The linearity of the proposed method was investigated in the range of 48.6-148.9µg/ml (r^2=0.999). The percent amount of drug estimated by all developed methods was nearly 100%, found to be in good agreement with label claim of marketed tablet formulation. The recovery study was carried out at three different levels and results were found to be satisfactory. The validation parameters like accuracy, precision, ruggedness,

linearity and range were studied for all developed methods and were found to be within limits.

Karthik A. [69] **et al.** developed and validate simple, fast and precise reverse phase, isocratic HPLC method was developed for the separation and quantification of pioglitazone and glimepiride in bulk drug and pharmaceutical dosage form. The quantification was carried out using inertsil ODS (250×4.6mm, 5µm) column and mobile phase comprised of acetonitrile and ammonium acetate (pH 4.5; 20mM) in proportion of 60:40 (v/v). The flow rate was 1.0 ml/min and the effluent was monitored at 230nm. The retention of pioglitazone and glimepiride were 7.0±0.1 and 10.2±0.1 min respectively. The method was validated in terms of linearity, precision, accuracy and specificity, limit of detection and limit of quantification. Linearity of pioglitazone and glimepiride were in the range of 2.0 to 200.0µ/ml and 0.5-50µg/ml respectively. The percentage recovery of both the drugs was 99.85% and 102.06% for pioglitazone and glimepiride respectively.

Sharma [70] **et al.** developed and validate simple reverse phase liquid chromatographic method has been developed and subsequently validated for simultaneous determination of pioglitazone and glimepiride in combination. The separation was carried out using a mobile phase of phosphate buffer (pH 4.5): acetonitrile (45:55 v/v) and using methanol as diluent. The column used was inertsil ODS (250×4.6mm i.d., 5µm) with flow rate of 1.0ml/min using UV detection at 225nm. The described method was linear over a concentration range of 5-50µg/ml and 5-25µg/ml for the assay of pioglitazone and glimepiride respectively. The retention times of pioglitazone and glimepiride were found to be 4.6 and 7.7min respectively. Results of analysis were validated statistically and by recovery studies. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for routine determination of pioglitazone and glimepiride bulk drug in pharmaceutical dosage form.

E. A. Rathod [71] **et al.** the present work proposed precise, accurate and validated HPLC and UV spectrophotometric methods for estimation of teneligliptin from its tablet dosage form. The UV spectrophotometric estimation includes calibration curve. Area under curve (AUC) and first order derivative method based on measurement of absorbance at a selected wavelengths using UV visible spectrophotometer with 1cm matched quartz cell and distilled water as a solvent. All UV spectrophotometric methods obeyed beer's-lambert's law in the concentration range of 10-70µg/ml, with correlation co-efficient

value less than 1. The chromatographic separation was achieved by isocratic mode with a mixture of methanol: phosphate buffer (pH 7.2) in the ratio of 70:30v/v as the mobile phase using shodex C18 column as stationary phase at flow rate of 1ml/min and detection wavelength of 244nm. The retention time was found to be 5.753min. The percent amount of drug estimated by all developed methods was nearly 100%, found to be in good agreement with label claim of marketed tablet formulation. The recovery study was carried out at five different levels and results were found to be satisfactory. The validation parameters like accuracy, precision, ruggedness, linearity and range were studied for all the developed methods were found to be within the limits.

K.P.R.Chowdary[72] **et al.** the stability indicating RP-HPLC method has been developed and validated for simultaneous estimation of metformin hydrochloride and teneligliptin in bulk and dosage forms. The involves separation on YMC C18 column (150mm×4.6mm×5µm particle size). The optimized mobile phase consists of phosphate buffer (pH 3) and acetonitrile (80:20 v/v) with a flow rate of 0.8ml/min and UV detection at 220nm. Retention time was 2.138min (metformin hydrochloride), 2.943 (teneligliptin), 5.075 pioglitazone. Linearity range was 9.98-600µg/ml (metformin hydrochloride), 0.51-24µg/ml (teneligliptin). Accuracy was in the range of 99.41-100.74% for both drugs. Precision was 0.8% and 0.9% for metformin hydrochloride and teneligliptin. LOD and LOQ are 0.72µg/ml and 2.40µg/ml for metformin hydrochloride, 0.15µg/ml and 0.51µg/ml for teneligliptin. The method developed is sensitive, accurate and precise. Retention time and run time were also less and hence the method is economical when applied to tablet assay, drug content was within 99.89-100.74% of labeled content. Forced degradation studies indicated the suitability of the method for stability studies.

Sohan S. Chitlange [73] **et al.** the simple, accurate precise and economical HPTLC and UV method has been developed and validated for the estimation of teneligliptin hydrobromide hydrate (THH) in bulk and tablet dosage form. The chromatographic method employed pre-coated silica gel 60F□□□ plates using toluene: methanol: triethylamine (8:2:0.2 v/v/v) as mobile phase. The plates were developed to a distance of 8.0cm at ambient temperature. Experimental condition such as band size, chamber saturation time, migration of solvent front, slit width, etc were critically studied and the optimum condition were selected. A TLC scanner set at 254nm was used for direct

evaluation of the chromatograms in reflectance/absorbance mode. The system was found to give good result for teneligliptin at Rf 0.51. The calibration plot was found to linear between concentrations range 0.5-3µg/ml band and r^2=0.9993. Method was validated as sper ICH guidelines. In stability testing, teneligliptin was found susceptible to alkali hydrolysis and oxidative degradation. Because the method could effectively separate the drug from its degradation products, it can be used stability indicating method. A UV spectrophotometric method was also developed using methanol as solvent at wavelength at 247nm. Beer's law was obeyed in the concentration range of 5-50µg/ml and r^2=0.9997. The proposed method was validated according to the ICH guidelines.

Shraddha Pawar[74]**et al.** a gradient method is developed for the quantitative determination of impurities of glimepiride and metformin hydrochloride in the combined pharmaceutical dosage form. The method is based on high performance liquid chromatography (HPLC) on a reverse phase column of waters symmetry C8, 5µm 4.6×250mm thermo stated at 50˚c, using a mobile phase of pentane sulfonic acid sodium salt buffer pH 3.5 and acetonitrile and evaluated for its ability to simultaneously establish the level of known impurities in glimepiride and metformin hydrochloride tablets. The method shows good resolution between glimepiride sulfonamide (GS), glimepiride urethane (GU), glimepiride 3-isomer (GI), metformin related compound A (MA), glimepiride (G), metformin hydrochloride (M), unknown impurities and formulation excipients of tablets. A gradient program with UV detection at 230nm is used to quantitate all components. The developed method is validated in term of specificity, linearity and range GS, GU, GI, MA, M and G. Accuracy using spiked level of impurities (80% to 120% of the specified limit), precision and ruggedness. Limit of quantitation is found to be 1.50µg/ml for M, 0.10µg/ml for G, 0.30µg/ml for MA, 0.24µg/ml for GS, 0.10µg/ml for GU and 0.22µg/ml for GI.

M Suchitra[75]**et al.** A rabid RP-HPLC method was developed and validated for simultaneous estimation of metformin and glimepiride, pioglitazone from pharmaceutical dosage forms. A sensitive chromatographic separation was accomplished on C18 column (100×4.6mm, 5µ) with mobile these consisting of methanol: phosphate puffer (pH3.6 adjusted with ortho phosphoric acid) in the ratio of 75: 25 v/v, at a flow rate of 1ml/min and monitored at 238nm. The developed method was validated in terms of accuracy, precision, linearity and limit of detection, limit of quantification, robustness and solution

stability. The proposed method can be used for the routine estimation of these drugs in combined pharmaceutical dosage form.

Shailesh V. Luhar [76] **et al.** a simple, rapid, precise and accurate reversed phase stability indicating RP-HPLC method was developed and validated for the simultaneous determination of teneligliptin hydrobromide hydrate from its associated main impurities and their degradation products. Separation was achieved on a shisedo C18 column, 5µm, 250mm×4.6mm i.e. column using a mobile phase consisting of acetonitrile: methanol: water (30:40:30 v/v/v) at flow rate of 1.0ml/min and UV detection at 246nm. The drugs are subjected to acid hydrolysis, alkaline hydrolysis, oxidative degradation and thermal degradation to apply force degradation testing. The linearity of the proposed method was investigated in the range of 50-300µg/ml (r^2=0.9996). The limit of detection was 2.78µg/ml and the limit of quantification was 8.45µg/ml respectively.

Prafulla M Patil [77] **et al.** a high performance thin layer chromatography method for determination of teneligliptin was developed and validated as per ICH guide lines. HPTLC separation was performed on aluminium plates precoated with silica gel 60F□□□ and methanol: toluene: triethylamine (1:3:1% v/v) volume as optimized mobile phase at detection wavelength at 245nm. The retardation factor (Rf) value for teneligliptin were 0.63 respectively. Accuracy for the marketed formulation teneza was found to be 98.31-100.51%. The percent relative standard deviation for repeatability and intermediate precision studies was found to be <2%. The propose development HPTLC method can be applied for identification and quantitative determination of teneligliptin respectively.

Amina A Abdelal[78]**et al.** the simple reversed phase high performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous estimation of rosiglitazone (ROS) and glimepiride (GLM) in combined dosage form and human plasma. The separation was achieved using a 150mm×4.6mm i.d., 5µm particle size C18 column. Mobile phase containing a mixture of acetonitrile and 0.02M phosphate buffer of pH 5 (60:40 v/v) was pumped at a flow rate of 1ml/min. UV detection was performed at 235nm using nicardipine as an internal standard. The method was validated for accuracy, precision, specificity, linearity and sensitivity. The developed and validated method was successfully used for quantitative analysis. The chromatographic analysis time was approximately 7min per sample with complete

resolution of ROS (Rt =3.7min), GLM (Rt =4.66min) and nicardipine (Rt =6.37min). Validation studies were performed according to ICH guidelines.

K. Neelima [79] **et al.** the simple, sensitive, linear, precise and accurate method by gradient reversed phase high performance liquid chromatography for the simultaneous estimation on metformin (MET), voglibose (VOG) and glimepiride(GLM) in bulk and in their combined tablet dosage form. The separation of the three drugs was based on the use of inertsil ODS 3V (150×4.6mm, i.e. 5µm) column in a gradient mode. Mobile phase consisted of 0.02M phosphate buffer adjusted to pH 2.5 using dilute orthophosphoric acid (solvent A) and acetonitrile (solvent B) was set with gradient programming for 18min and was delivered at 1ml/min flow rate and effluents are achieved with variable wavelength, photodiode array detector at 230nm. The retention time of MET, VOG and GLI were found to be 2.423, 8.191 and 11.708 respectively. The percentage assay of MET, VOG and GLI at concentration ranges of 200-600µg/ml, 0.08-0.24µg/ml and 0.8-2.4µg/ml with the regression coefficient of 0.999 for all the three drugs precise with (%relative standard deviation <2). The limit of detection MET, VOG and GLI was found to be 0.05µg/ml, 0.004µg/ml, 0.002µg/ml and limit of quantification for MET, VOG and GLI was found to be 1.5µg/ml, 0.012µg/ml and 0.006µg/ml respectively.

3. DRUG PROFILE

TENELIGLIPTIN HYDROBROMIDE HYDRATE: [80-83]

1. Chemical profile

Chemical name/ IUPAC name : {(2s,4s)-4-(4-(3-methyl-1-phenyl-1H-pyrazol-5-yl)-1-piperazinyl)-2-pyrrolidinyl)-3-thiazolidinyl hydrobromide,(2:5),hydrate.

Molecular formula :(C□□H□□N□OS) 2.5HBr.xH□O

Molecular mass : 1275.74 g/mol

Molecular structure :

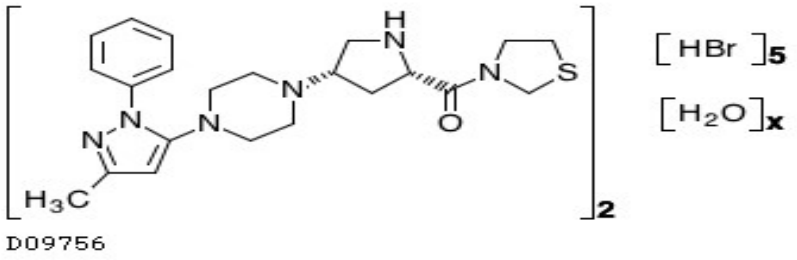

Fig. 3: Structure of teneligliptin hydrobromide hydrate

2. Pharmacokinetic data

Bioavailability : 63-85%

Protein Binding : 78-80%

Metabolism : CYP3A4, a cytochrome P450 isoenzyme andflavin Containing monooxygenases (FMO1 and FMO3) Play Major roles in the metabolism of teneligliptin.

Biological half life : 8.43-24.2 hrs

Excretion : kidney (34.4%) renal (65.6%)

Route of administration : Oral

Solubility : DMSO, methanol, water

3. Mechanism of action

Teneligliptin inhibits human dipeptidyl peptidase-4 (DPP-4) enzyme activitywith the IC□□=1 nM, more than 150 fold selectivity against DPP-8 and DPP-9 which Suggested little of target skin lesion side effect.

4. Dosing

Adult dose: the usual adult dosage is 20mg of teneligliptin orally administered once a daily.

5. Side effects

- Hypoglycemia
- Constipation
- Feeling of abdomen enlarged
- Abdominal discomport
- Nausea
- Abdominal pain
- Meteorism
- Eczema
- Rash
- Pruritus
- Dermatitis

GLIMEPIRIDE [84-87]

1. Chemical profile

Chemical name/ IUPAC name : 3-ethyl-4-methyl-2-oxo-N-(2-{4-[({[(1r,4r)-4-methylcyclohexyl]-c-hydroxycarbonimidoyl}amino)sulfonyl]phenyl}ethyl)-2,5-dihydro-1H-pyrrole- 1-carboximidic acid

Molecular formula : C□□H□□N□O□S

Molecular mass : 490.617 g/mol

Molecular structure :

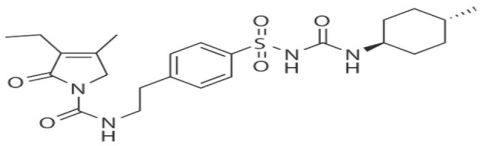

Fig. 4: Structure of glimepiride

2. Pharmacokinetic data

Bioavailability : 100%

Protein binding : 99.5%

Metabolism : complete hepatic (1st stage through CYP2C9)

Biological half-life : 5-8 hrs

Excretion : urine (60%) feces (40%)

Routes of administration : oral

Solubility : soluble in ethanol, DMSO, methanol and water

3. Mechanism of action

Glimepiride acts as an insulin secretagogue. It lowers blood sugar by stimulating the release of insulin by pancreatic beta cells and by inducing increased activity of intracellular insulin receptors.

Not all secondary sulfonylureas have the same risks of hypoglycemia. Glibenclamide (glyburide) Is associated with an incidence of hypoglycemia of up to 20-30%to as low as 2% to 4% with glimepiride.

4. Dosing

Adult dosage: The recommended starting dose 1mg or 2mg taken once per day with breakfast or the first main meal of the day

Child dosage: glimepiride is not recommended for people under 18 years old because it may affect body weight and cause low blood sugar.

5. Side effects

Low blood sugar level symptoms may include:

- Trembling or shaking
- Nervousness or anxiety
- Irritability
- Sweating
- Dizziness
- Nausea
- Weakness
- Weight gain

4. AIM AND OBJECTIVES

For the simultaneous estimation of drugs present in the multi-component dosage forms, HPLC and HPTLC methods are considered to be most suitable. These methods are powerful, extremely precise, accurate, sensitive, specific, linear and rapid in analyzing the sample. The chromatographic methods was found to be most suitable than the other methods, due to its very high sensitivity.

The present study is to develop and validate RP-HPLC and HPTLC methods for the identification and quantification by simultaneous estimation of teneligliptin hydrobromide hydrate and glimepiride in tablet dosage form.

From the extensive literature survey revealed several methods for estimation of individual drugs. There is no RP-HPLC and HPTLC methods were reported for the simultaneous estimation of teneligliptin hydrobromide hydrate and glimepiride in combined dosage form. The proposed methods were lacking the ICH standards.

So the major objectives of the present study are;

- To reduce the flow rate as compared with previously reported method in literature.
- To carry out simultaneous estimation by using different chromatographic conditions.
- To develop and validate a HPLC and HPTLC method for the simultaneous estimation of the drug substances.

Based on the above facts, RP-HPLC and HPTLC methods were developed for the simultaneous estimation of teneligliptin hydrobromide hydrate and glimepiride.

5. PLAN OF WORK

The work plan was to develop RP-HPLC and HPTLC methods for the simultaneous estimation of teneligliptin hydrobromide hydrate and glimepiride.

HPLC and HPTLC:

The work plan was divided into two phases.

Phase I:

Optimization of chromatographic conditions

- Selection of wavelength
- Selection of initial separation conditions
- Selection of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate)
- Nature of the stationary phase
- Selection of separation method

Phase II:

Validation of the method

The developed method were proposed to be validated using the various validation parameters such as,

- Accuracy
- Precision
  - ✓ Inter day
  - ✓ Intraday
  - ✓ repeatability
- Linearity
- Limit of detection
- Limit of quantification
- Robustness
- Ruggedness
- System suitability

6. METHODOLOGY

HPLC METHOD DEVELOPMENT

MATERIALS AND INSTRUMENTS USED

1) **Drug standard and sample**

Teneligliptin hydrobromide hydrate and Glimepiride drug (pure) was received as a gift sample from micro labs Ltd Bangalore.

Tablet sample were selected for the method development

Each film coated tablet contains:

- Teneligliptin hydrobromide hydrate: 20mg
- Glimepiride : 1mg

2) **Chemicals and solvents**
   - Acetonitrile HPLC grade
   - Methanol HPLC grade
   - Ortho phosphoric acid AR grade
   - HPLC grade water was prepared by using Millipore MilliQ water purification system

3) **Instruments**
   - Elico pH meter L1 127
   - SPD-M20A prominence diode array detector
   - Shimadzu 1600LC-UV spectrophotometer
   - Sonica ultrasonic cleaner
   - Solvent filtration unit- Millipore
   - Shimadzu electronic balance AY 220
   - Shimadzu prominence HPLC
     - ✓ Pump- Prominence LC- 20AT
     - ✓ Column- Phenomenex Luna 5µ C18 (2) 100A, (250mm×4.6×i.d. 5µ)
     - ✓ Inject- Rheodyne 7725*i* with 20µl loop
     - ✓ Detector- Photo diode array detector

METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITION

The present work is the development of the RP-HPLC method for the simultaneous estimation of Teneligliptin hydrobromide hydrate and Glimepiride in tablet dosage form.

a) Selection of wavelength:

The wavelength for the analysis of Teneligliptin hydrobromide hydrate and Glimepiride was selected by injecting several times of mixed standards of 10 µg/ml of Teneligliptin hydrobromide hydrate and 10 µg/ml of Glimepiride, for optimum detection of both the drugs. Since both Teneligliptin hydrobromide hydrate and Glimepiride have shown maximum absorbance at 246nm, finally which was selected as an optimum wave length for the estimation method.

b) Selection of method for separation:

Proper selection of the method depends upon the nature of the sample (ionic/ionisable/neutral molecule), its molecular weight and solubility. The drug selected in the present study is polar in nature and hence the reverse phase HPLC was selected for the initial separations because of its simplicity and suitability.

c) Initial chromatographic conditions:

Chromatographic condition: 1

Stationary phase	: Phenomenex C18 column
Mobile phase	: Solvent A – water
	Solvent B - acetonitrile
Solvent ratio	: 50: 50 (A: B)
Detection Wavelength	: 246 nm
Flow rate	: 1.0 ml/min

Sample size : 20 µl

Temperature : 25ºC

At the above chromatographic condition Teneligliptin hydrobromide hydrate and Glimepiride was eluted at a retention time of 7.33 min and 13.32 min. The peaks observed were broad and asymmetric hence not selected further for method development.

Chromatographic Condition – 2

Stationary phase : Phenomenex C18 column

Mobile phase : Solvent A – water (pH adjusted to 4.0 with Orthophosphoric acid) Solvent B - acetonitrile

Solvent ratio : 50: 50 (A: B)

Detection Wavelength : 246 nm

Flow rate : 1.0 ml/min

Sample size : 20 µl

Temperature : 25ºC

Teneligliptin hydrobromide hydrate was eluted at retention time of 6.20 min with peak splitting, Glimepiride at retention time 8.35 min with peak splitting hence not selected further for method development.

Chromatographic Condition – 3

Stationary phase : Phenomenex C18 column

Mobile phase : Solvent A – water (pH adjusted to 4.0 with Orthophosphoric acid) Solvent B - acetonitrile

Solvent ratio : 30: 70(A: B)

Detection Wavelength : 246 nm

Flow rate : 1.0 ml/min

Sample size : 20 µl

Temperature : 25ºC

Teneligliptin hydrobromide hydrate was eluted at retention time 6.33 min with peak tailing and Glimepiride was eluted at 12.33 min. Peak tailing was observed hence not selected.

Chromatographic Condition – 4

Stationary phase : Phenomenex C18 column

Mobile phase : Solvent A – water (pH adjusted to 3.0 with Orthophosphoric acid) Solvent B - acetonitrile

Solvent ratio : 40: 60 (A: B)

Detection Wavelength : 246 nm

Flow rate : 1.0 ml/min

Sample size : 20 µl

Temperature : 25ºC

Teneligliptin was eluted at retention time 6.44 min and Glimepiride was eluted at 10.2 min with peak broadening hence not selected.

Chromatographic Condition – 5

Stationary phase : Phenomenex C18 column

Mobile phase : Solvent A – water (pH adjusted to 3.5 with Orthophosphoric acid) Solvent B - acetonitrile

Solvent ratio : 30: 70 (A: B)

Detection Wavelength : 246 nm

Flow rate : 1.0 ml/min

Sample size : 20 µl

Temperature : 25^{o}C

Teneligliptin hydrobromide hydrate was eluted at retention time 6.44 min and Glimepiride was eluted at 12.2 min with peak broadening hence not selected.

OPTIMIZATION OF SEPARATION CONDITIONS

Effect of pH:

Table 4: Effect of PH

Drug	pH	Retention time (min)	Observation
TENE	4.0	6.2	Fronting
	3.5	6.4	Tailing effect
	3.0	5.8	Good
GLI	4.0	12.3	Peak broadening
	3.5	12.2	Splitting
	3.0	9.4	Good

Effect of ratio of mobile phase:

The mobile phase of acetonitrile: phosphate buffer in various ratios, 50:50, 60:40, 70:30. 65:35 and 80:20 were tried and the chromatograms were recorded at 246nm with a flow rate of 1ml/min. At the ratio of 65:35 of acetonitrile: phosphate buffer was selected (pH adjusted with 3.0 with orthophosphoric acid) as an ideal ratio for the estimation of Teneligliptin hydrobromide hydrate and Glimepiride.

The standard solution was chromatographed for 20min, using 65% acetonitrile and phosphate buffer solution of different pH ranges 4.0, 3.5, 3.0 at 246nm, the mobile has the flow rate of 1ml/min using phenomenex C18 column as stationary phase for

Teneligliptin hydrobromide hydrate and Glimepiride determination and pH 3.0 was selected as the ideal pH for the separation of the drugs.

Table 5: Effect of mobile phase

Drug	Ratio (%v/v)	Retention time (min)	Observation
TENE	50:50	4.6	Broad
	60:40	6.3	Tailing effect
	65:35	5.8	Good
GLI	50:50	8.5	Little broad
	60:40	12.4	Splitting
	65:35	9.4	Good

Effect of flow rate:

Keeping the mobile phase ratio 65:35 (acetonitrile: phosphate buffer) were used and the chromatograms were recorded at a flow rate 1ml/min. At this flow rate, the peaks were sharp with good resolution. So 1ml/min was kept constant for the analysis (flow rate 0.9ml/min, 1.1ml/min up to 1.3ml/min were also tried, but did not give any satisfactory results).

Mobile phase:

Prepare a filtered and degassed mixture of acetonitrile: phosphate buffer pH adjusted to 3.0 with orthophosphoric acid (65:35) was used as a mobile phase.

Fixed chromatographic conditions:

Stationary phase	: Phenomenex C18 column
Mobile phase	: Solvent A – phosphate buffer (pH adjusted to 3.0 with Orthophosphoric acid) solvent B- acetonitrile
Solvent ratio	: 35:65 (A: B)
Detection Wavelength	: 246 nm
Flow rate	: 1.0 ml/min
Sample size	: 20 µl
Temperature	: 25^{o}C

Teneligliptin was eluted at 5.8 min and Glimepiride was eluted at 9.41 min with perfect peak properties and hence selected for further studies.

Estimation of Teneligliptin hydrobromide hydrate and Glimepiride:

Estimation of Teneligliptin hydrobromide hydrate and Glimepiride in tablet dosage forms by RP-HPLC method was carried out by standard calibration method.

Preparation of standard solution:

Weighed accurately 10 mg of Teneligliptin hydrobromide hydrate and was dissolved in methanol and made up to 10ml. 0.1ml of this stock solution was diluted to 10ml to get solution A. Similarly 1mg of Glimepiride was dissolved in methanol and made up to 20ml and 0.1ml of this stock solution was diluted to 10ml to get a solution B.

Mix 0.1ml of each stock solution in 10ml standard flask made up to 10ml methanol with final concentration solution of Teneligliptin hydrobromide hydrate and Glimepiride respectively.

Aliquots of mixed standard solutions of **0.05ml, 0.10ml, 0.15ml, 0.20ml, 0.25ml of TENE and GLI** were diluted in mobile phase to get a final concentration of **5, 10, 15, 20, 25 µg/ml** of Teneligliptin hydrobromide hydrate and **0.25, 0.5, 0.75, 1, 1.25 µg/ml** of Glimepiride. All the solutions were sonicated for 20 minutes before injection.

Preparation of sample solution:

Twenty tablets of each containing Teneligliptin hydrobromide hydrate-20mg, and Glimepiride 1mg were weighed, and crushed into fine powder. A quantity of powder equivalent to 20mg of Teneligliptin was weighed and dissolved in 5 ml of mobile phase (4:6) and sonicated for 15 min. Then the volume was made up to 10 ml with mobile phase ratio and filtered through whatmann filter paper. The aliquots of **0.05ml, 0.10ml, 0.15ml, 0.20ml, 0.25ml of TENE and GLI** the final sample solution was prepared.

Recording chromatogram

After optimization of chromatographic conditions mentioned above, a study baseline for about 30 min was recorded. After the stabilization of the baseline, 100µg/ml of the standard solutions were injected and chromatograms were recorded until the reproducibility of the peak areas were found satisfactory and finally 10µg/ml of the standard solutions of the individual samples of Teneligliptin hydrobromide hydrate and Glimepiride were recorded. The standard solution containing 5-25 µg/ml of Teneligliptin hydrobromide hydrate and 0.25-1.25 µg/ml of Glimepiride were injected and chromatograms were recorded.

Retention time of Teneligliptin hydrobromide hydrate and Glimepiride were found to be 5.8 and 9.4min respectively **Fig.5.**

The procedure was repeated as both sample and standard solution. The peak purity, UV spectrum and peak profile of Teneligliptin hydrobromide hydrate was shown in **Fig. 14, 15 and 16** and foe Glimepiride was shown in **Fig. 17, 18 and 19** respectively.

Calibration curves were plotted using peak area against concentration of corresponding standard solutions. Peak areas of the sample chromatograms were recorded and the amount of teneligliptin hydrobromide hydrate and glimepiride were calculated.

VALIDATION OF THE METHOD

Validation is the process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

Significance of method validation:

The quality of analytical data is a key factor in the success of a drug development program. The process of method development and validation has a direct impact on the quality of these data.

Analytical validation is a very important feature of any package of information submitted to international regulatory agencies in support of new product marketing or clinical trial applications. A through method development can almost rule out all potential problems, at the same time, a through validation programme can address the most common ones and provide assurance to the intended purpose (can be used with 100% confidence). In other words, a through validation can fulfil all the technical and regulatory objectives. A direct consequence and most significant out come from any method validation exercise is the development of meaningful specifications can be predicted upon the use of validated analytical procedures that can assess changes in drug substance or drug product during its life time.

Analytical characteristics listed below may not be applicable to every test procedure or every particular material. It will mostly depend on the purpose of which the procedure is required, however, these following aspects of validation should be given due importance.

a) Accuracy:

Accuracy method was determined by recovery experiments. The reference standards of the respective drug were added to the sample solution, (10 µg/ml of Teneligliptin hydrobromide hydrate and 0.50 µg/ml of Glimepiride) at the level of 50%, 100% and 150%. These were further diluted by procedure as followed in the estimation of formulation. The concentration of drug present in the resulting sample solution was determined by using assay method.

b) Precision:

The precision of the developed method was determined in terms of intermediate precision (intra-day and inter-day) and repeatability. Teneligliptin hydrobromide hydrate (15 µg/ml) and Glimepiride (0.75 µg/ml) were analysed in six times during the same day (intra-day precision) and three consecutive days (inter-day precision). The %RSD values of intra-day, inter-day and repeatability studies for Teneligliptin hydrobromide hydrate and Glimepiride showed that the precision of the method was satisfactory.

c) Linearity and range:

From the standard stock solutions, a suitably mixed standard solution was prepared. Teneligliptin hydrobromide hydrate and Glimepiride were found to be linear in the range of 5 to 25 µg/ml and 0.25 to 1.25 µg/ml respectively. The solutions were examined by the assay procedure. The calibration curve was plotted by using peak area Vs concentration of the standard solution. From the calibration curve, the slope and intercept were calculated.

d) Limit of detection (LOD) and Limit of quantification (LOQ):

The LOD and LOQ was separately determined and reported in **Table 13,** based on the calibration curve of standard solution. The residual standard deviation of the regression line or the standard deviation of y- intercepts of regression lines may be used to calculate LOD and LOQ. LOD= $3.3 \times \sigma/S$ and LOQ= $10 \times \sigma/S$, where, σ is the standard deviation of y- intercepts of regression line and S is the slope of the calibration curve.

LOD is the smallest concentration of the analyte that can be detected and gives the measurable response (signal to noise ratio of 3). The signal to noise ratio were performed by comparing by measured signal of known low concentration of drug. LOQ is the smallest concentration of analyte that can be accurately quantified (signal to noise ratio 10).

e) Specificity:

The specificity of the RP-HPLC method was determined by complete separation of Teneligliptin hydrobromide hydrate and Glimepiride with parameters like retention time (Rt), resolution (Rs) and tailing factor (T), peak purity curve and peak purity index.

Tailing factor for peaks of teneligliptin hydrobromide hydrate and glimepiride less than 2% and resolution was satisfactory. The peaks obtained for were sharp and have clear baseline separation. The peak purity studies were performed to prove that the method is specific in nature.

f) Ruggedness:

It expresses the precision within laboratory variations like different days, different analyst and different equipments. Ruggedness of the method was assessed by spiking the standard concentrations of Teneligliptin hydrobromide hydrate (15 µg/ml) and Glimepiride (0.75 µg/ml), 6 times in two different days with different analyst.

g) Robustness:

In order to demonstrate the robustness of the method, the following optimized conditions were slightly varied.

1) ± 2% in ratio of acetonitrile in mobile phase,
2) ± 0.2ml of flow rate
3) ± units in the pH of buffer
4) ± 2nm wavelength

The separation factor, retention time and peak symmetry were than calculated. The deviation among the results obtained is well within the limits. Hence the method is robust.

h) System suitability studies:

The system suitability studies were carried out as specified in ICH. These parameters include number of theoretical plates, HETP, column efficiency, resolution and capacity factor.

HIGH PERFORMNCE THIN LAYER CHROMATOGRAPHY (HPTLC) METHOD DEVELOPMENT

a) Drug standard and sample:

Teneligliptin hydrobromide hydrate and Glimepiride drug (pure) was received as a gift sample from micro labs Ltd Bangalore.

Tablet sample were selected for the method development

Each film coated tablet contains:

- Teneligliptin hydrobromide hydrate: 20mg
- Glimepiride : 1mg

b) Chemicals and solvents used:

The selected solvent must give some ideal properties like, the drug should be stable in the selected solvent. Hence methanol was selected as the solvent for the drug. Other solvents used were of analytical grade.

c) Selection of detection wavelength:

The sensitivity of the HPTLC is depending on the wavelength selected and the UV detector. An ideal wavelength is the one that gives maximum absorbance and good response for the drug detected at lower concentration also. The drugs were scanned under UV spectrum between 200-400 nm and the detection was carried out in absorbance mode at 246 nm. It was selected as detection wavelength for the selected mobile phase.

d) Selection of mobile phase:

Various mobile phases at various ratios were tried for the separation of the drugs. The R_f values were calculated for the methods tried and a mobile phase with optimum separation was selected.

Table 6: various mobile phase system were tried for optimization of mobile phase

Mobile Phase	Rf value	Observation

	GLI	TENE	
Toluene: Chloroform: Ethanol: Diethyl amine (4:4:1:1 v/v/v/v)	0.28	0.59	Asymmetrical peak
Toluene: Chloroform: Ethanol: diethyl amine (4.5:3.5:1:1 v/v/v/v)	0.43	0.69	Broad peak
Toluene: Chloroform: Ethanol (3:6:1 v/v/v)	0.35	0.59	Broad peak
Toluene: Methanol: Triethyl amine (2:2:1 v/v/v)	0.59	0.64	Merged peak
Toluene: Methanol: Triethyl amine (1:3:1 v/v/v)	0.49	0.60	Symmetrical peak

Optimization

Fixed experimental parameters

Injection : Linomat 5

Detection : CAMAG TLC scanner

Information:

Chamber type : Twin trough chamber 20x10cm

Stationary phase : Pre - coated silica gel GF aluminium sheets TLC plate

Mobile phase : Methanol: Toluene: Triethyl amine (1:3:1v/v/v)

Chamber saturation : 20 min

Band length : 6.0 mm

Application position : 10.0 mm

Solvent front position : 80.0 mm

Instrument:

Number of track : 9

Position of first track X : 10.0 mm

Distance between tracks : 10.0mm

Scan start position Y : 5.0 mm

Scan end position Y : 75.00mm

Slit dimensions : 6.00 x 0.45 mm, micro

Measurement table:

Wavelength : 246 nm

Lamp : D2

Measurement type : Remission

Measurement mode : Absorption

Optical filter : Second order

Detector mode : Automatic

Preparation of standard solution:

Mixed stock standard solution containing 20 mg of TENE and 1mg of GLI in 10 ml methanol. Mixed stock standard solution was further diluted with methanol to obtain working standard solutions in a concentration range of 2000-20000(ng/spot) for TENE and 100-1000 (ng/spot) for GLI.

Preparation of sample solution:

For analysis of tablet dosage form, twenty tablets, each containing 20mg Teneligliptin hydrobromide hydrate and 1 mg Glimepiride, were weighed and their average weight was calculated. The tablets were finely powdered and powder equivalent to 20mg of TENE was accurately weighed and transferred into 10 ml of volumetric flask containing 5 ml of methanol, Sonicated for 30 min. The solution was filtered through Whattman No 41 filter paper and the residue was washed with methanol. The volume of the filtrate was adjusted to 10 ml with the same solvent. This above solution was further diluted with methanol to get the concentrations of 4000, 8000and 16000 (ng/spot) for TENE and 200, 400 and 800 (ng/spot) for GLI.

VALIDATION OF HPTLC METHOD

a) Accuracy:

Accuracy of the method was determined by recovery experiments. The reference standards of the respective drug were added to the sample solution 8000 (ng/spot) of Teneligliptin hydrobromide hydrate and 400 (ng/spot) of Glimepiride at the level of 50%, 100% and 150%. These were further diluted by procedure as followed in the estimation of formulation. The concentrations of the drugs present in the resulting sample solution were determined by using assay method.

b) Linearity and range:

From the standard stock solutions, a suitably mixed standard solution was prepared. Teneligliptin hydrobromide and Glimepiride were found to be linear in the range of 2000 to 20000 (ng/spot) and 100 to 1000 (ng/spot) respectively. The solutions were examined by the assay procedure. The calibration curve was plotted using peak area Vs concentration of the standard solution. From the calibration curve, the slope and intercept were calculated.

c) Precision:

Precision of the method was determined by:

Intra-day precision

Inter-day precision

Repeatability

a) Intra-day Precision:

Intra-day precision was found out by carrying out the analysis of the standard drug solutions at concentration of 4000-16000 (ng/spot) of TENE and 200-800 (ng/spot) of GLI for three times on the same day. The Percentage RSD was calculated.

b) Inter-day precision:

Inter-day precision was found out by carrying out the analysis of the drug solution at a concentration of 4000-16000 (ng/spot) of TENE and 200-800 (ng/spot) of GLI for three different days and the percentage RSD was calculated.

c) Repeatability:

Repeatability of measurement of the peak area was determined by spotting 8000(ng/spot) TENE and 400 (ng/spot) GLI of drug solution on a pre-coated TLC plate. The separated spots were scanned five times without changing the position of the plate and the percentage RSD was calculated.

d) Limit of Detection (LOD) and Limit of Quantification (LOQ):

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a standard which can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ were experimentally verified by the known concentration of a standard solution of Teneligliptin hydrobromide hydrate and Glimepiride until the average response approximately 3 or 10 times the standard deviation of the responses for five replicate determinations.

e) Specificity:

It was observed that other constituent's presents in the formulation did not interfere either with the peak of Teneligliptin hydrobromide hydrate and Glimepiride. Therefore the method was specific. The overlay spectrum of the standard Teneligliptin hydrobromide hydrate and Glimepiride spots present in the samples were found to be similar or overlap. The peak purity of the Teneligliptin hydrobromide hydrate and Glimepiride was assessed by comparing the spectra at three different levels, viz. peak start, and peak apex and peak end positions of the spot.

f) Robustness of the method:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small changes in the mobile phase composition, mobile phase volume and duration of mobile phase saturation and the effects on the results were examined.

g) Ruggedness:

It expresses the precision within laboratory variations like different days, different analyst, and different equipments. Ruggedness of the method was assessed by spiking the standard concentrations of TENE 8000 (ng/spot) and GLI 400 (ng/spot), five times in two different days with different analyst.

7. RESULTS AND DICUSSION

HPLC

A RP-HPLC method was developed for the simultaneous estimation of Teneligliptin hydrobromide and Glimepiride in tablet dosage form, which can be conveniently employed for routine quality control in pharmaceutical dosage forms.

Fixed chromatographic conditions:

Stationary phase	: Phenomenex C18 column
Mobile phase	: Solvent A – phosphate buffer (pH adjusted to 3 with Orthophosphoric acid) solvent B- acetonitrile
Solvent ratio	: 35:65 (A: B)
Detection Wavelength	: 246 nm
Flow rate	: 1.0 ml/min
Sample size	: 20 µl
Temperature	: 25^{o}C

Teneligliptin was eluted at 5.8 min and Glimepiride was eluted at 9.41 min with perfect peak properties and hence selected for further studies.

<Chromatogram>

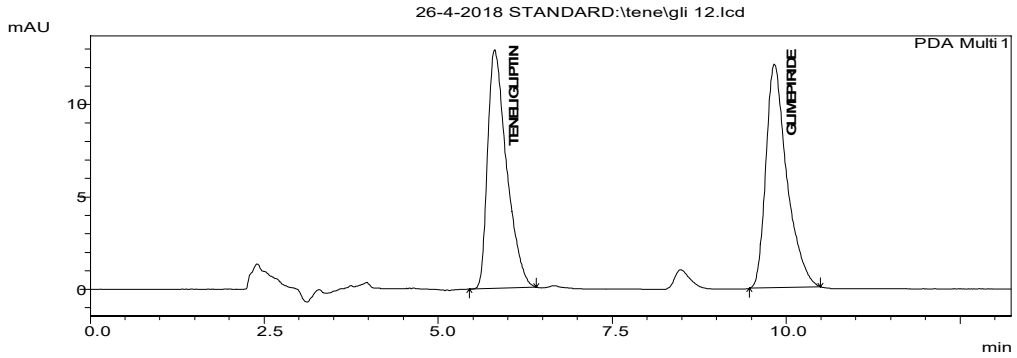

Fig.5: Typical chromatogram obtained for the Teneligliptin hydrobromide hydrate and Glimepiride.

1. Linearity:

Teneligliptin hydrobromide hydrate and Glimepiride were found to be linear in the range of 5 to 25 µg/ml and 0.25 to 1.25 µg/ml respectively.

The correlation coefficient of Teneligliptin hydrobromide hydrate and Glimepiride were found to be 0.998 and 0.996 respectively. The linearity range of Teneligliptin hydrobromide hydrate and Glimepiride were shown in **Table 7 and 8** respectively. The calibration curves were plotted as peak area Vs concentration of the standard solutions (**Fig**. **6 and 7)**.

The calibration graph showed linear response over the range of 5 to 25 µg/ml for TENE and 0.25 to 1.25 µg/ml of GLI. The range demonstrates that the method is linear outside the limits of expected use.

Table 7: Linearity range of Teneligliptin hydrobromide hydrate

S.NO	Conc. of TENE (µg/ml)	Peak area*
1	5	132821
2	10	230885
3	15	351956
4	20	480892
5	25	620783

*mean of five observations

Calibration curve of teneligliptin

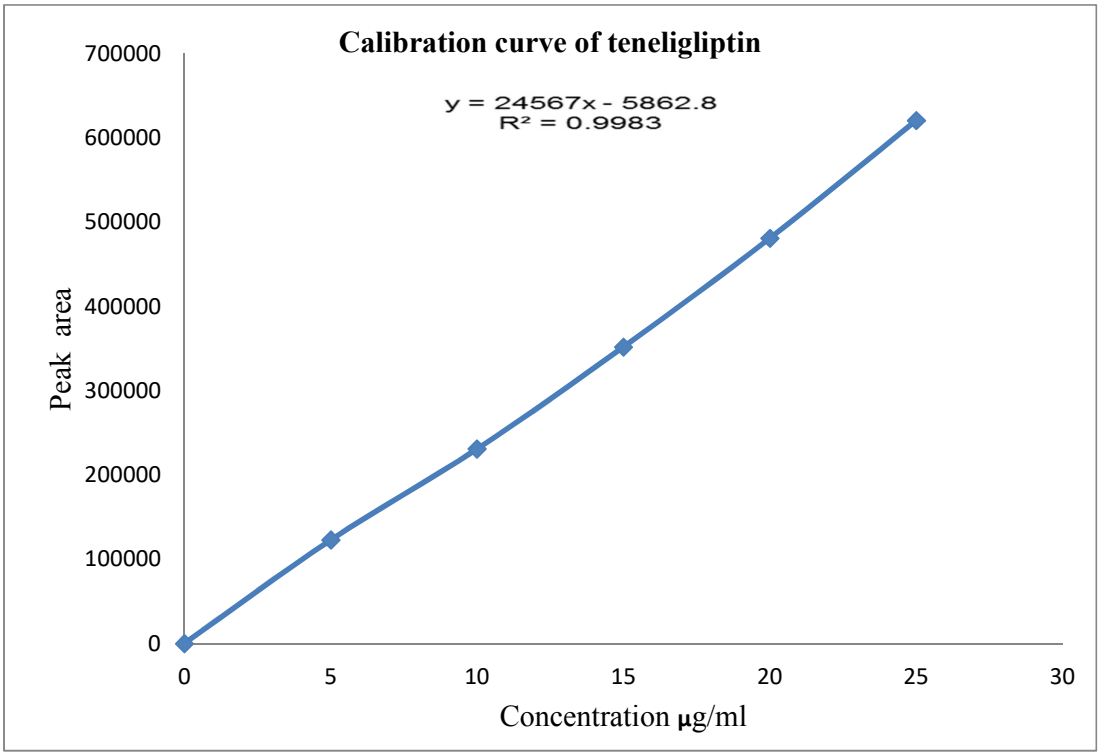

Fig.6: Calibration curve of Teneligliptin hydrobromide hydrate

Table 8: Linearity range of Glimepiride

S.NO	Conc. of GLI (µg/ml)	Peak area*
1	0.25	19705
2	0.5	37846
3	0.75	56171
4	1	71068
6	1.25	85898

*mean of five observations

Calibration curve of glimepiride

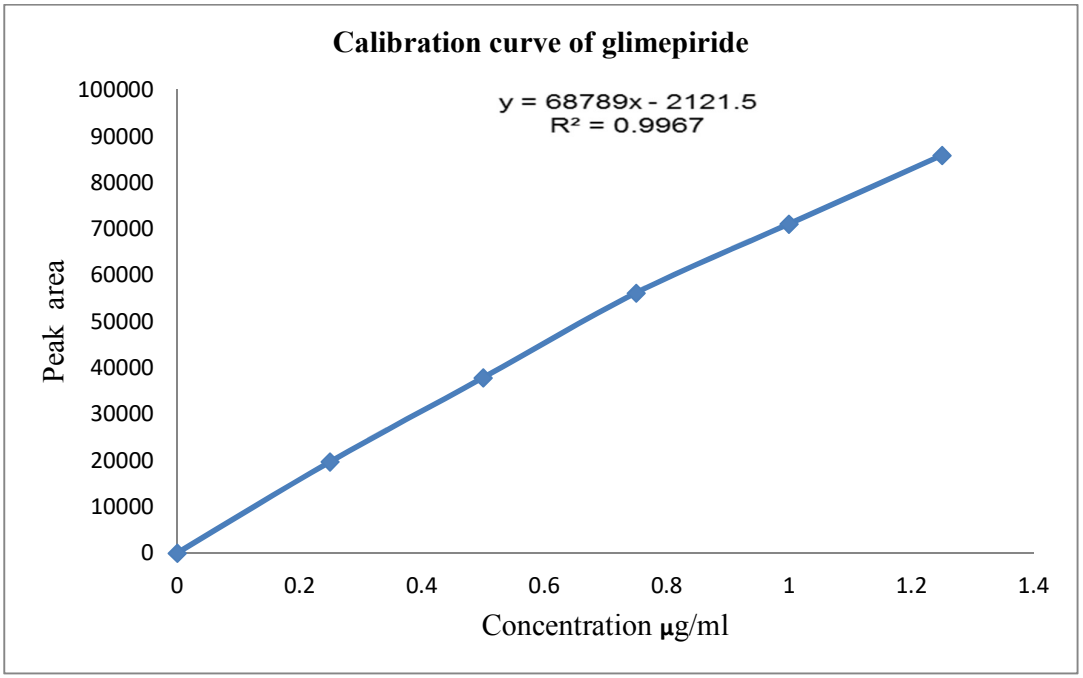

Fig. 7: Calibration curve of Glimepiride

Acceptance criteria

The results complied with an acceptance criteria since the linearities were found to be with in the specified limit of correlation co-efficient ie, 0.99.

2. Accuracy (Recovery studies):

The accuracy of the method was determined by recovery experiments. A known quantity of the pure drug was added to the pre-analyzed sample formulation at 50%, 100% and 150% levels. The recovery studies were carried out 6 times of each level and the percentage recovery and percentage relative standard deviation were calculated and given in **Table 9.** The percentage recovery of Teneligliptin hydrobromide hydrate and Glimepiride were found to be in the range of 98.83 to 99.83% and 99.60 to 99.90% respectively.

Table 9: Accuracy (Recovery studies):

Drug	Label claim mg/tab	Spike Level (%)	Amount of drug added (µg/ml)	Amount of drug recovered (µg/ml)	Percentage Recovery (%)	%RSD*
TENE	20	50	15	14.92	98.90	0.40
		100	20	19.8	98.83	0.36
		150	25	24.90	98.93	0.40
GLI	1	50	0.75	0.749	99.60	0.27
		100	1	0.98	99.89	0.22
		150	1.25	1.249	99.90	0.28

*mean of five observations

From the data obtained, it was observed that the recoveries of standard drugs were found to be accurate and within the specified limits.

3. Precision:

The precision of the method was determined by studying reproducibility and repeatability. The area of drug peaks and percentage relative standard deviation of intraday and inter day were calculated and presented in **Table 10 and 11**. The results revealed that the developed method was found to be reproducible in nature.

Table 10: Intraday studies

No. of Injection	Conc. of TENE (µg/ml)	Peak Area*	% RSD*	Conc. of GLI (µg/ml)	Peak Area*	% RSD*
6	15	353277.0	0.39	0.75	56880.0	0.25

*mean of five observations

Acceptance criteria

The results obtained complied with acceptance criteria since percentage relative standard deviation of peak areas of TENE and GLI were found to be with in the limit ie, NMT 2%.

Table 11: Inter day studies

Day	Conc. Of TENE (µg/ml)	Peak Area*	% RSD*	Conc. of GLI (µg/ml)	Peak Area*	% RSD*
DAY1	15	353270.5	0.36	0.75	56532.00	0.23
DAY2	15	353112.80	0.35	0.75	56840.30	0.24
DAY3	15	353412.5	0.35	0.75	56896.20	0.25

*mean of five observations

Acceptance criteria

The results obtained complied with acceptance criteria since percentage relative standard deviation of peak areas of TENE and GLI were found to be with in the limit ie, NMT 2%.

Table 12: Repeatability

Conc. of TENE (µg/ml)	Peak Area*	% RSD*	Conc. of GLI (µg/ml)	Peak Area*	% RSD*
15	353277.0	0.39	0.75	56880.16	0.26

*mean of five observations

Acceptance criteria

The results obtained complied with acceptance criteria since percentage relative standard deviation of peak areas of TENE and GLI were found to be with in the limit ie, NMT 2%.

4. LOD and LOQ:

The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3.3). The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10).

Table 13: LOD and LOQ

Parameter	TENE (µg/ml)	GLI (µg/ml)
LOD	1.320	0.903
LOQ	3.561	2.736

5. Ruggedness:

The sample was analyzed by a different chemist and same instruments on different days have been performed. The method is rugged since the deviation among the results obtained by two chemists on a different day was within the limits i.e., NMT 2%.

Table 14: Ruggedness studies

Drug name	Concentration (µg/ml)	Peak area*	%RSD*
Day-1 analyst-1			
TENE	15	357245.00	0.82
GLI	0.75	56247.16	0.64
Day-2 analyst-2			
TENE	15	358142.5	0.79
GLI	0.75	56392.00	0.59

*mean of five observations

6. Robustness:

The robustness studies were performed for the standard solutions and were presented in **Table 15**. The assay values were within the limits thus the developed method is robust.

Table 15: Robustness studies

Parameters	Modifications	TENE Recovery (%)	GLI Recovery (%)
pH	3.5	98.96	98.53
	4.0	99.92	99.84
Detection wavelength (nm)	244	99.89	99.33
	248	98.79	98.92
Flow rate (ml/min)	1.1	99.93	99.93
	0.9	98.76	98.89

*mean of five observations

7. System suitability studies:

The system suitability studies were performed for the standard solutions and are presented in **Table 16**. The values obtained demonstrate the system suitability for the analysis of the above drug combination.

Table 16: System suitability studies

Parameters	TENE	GLI
No. Of Theoretical Plates	5069	5909
Tailing Factor	0.98	1.02
HETP	20.27	23.63
LOQ	3.57 ng/ml	2.73 ng/ml
LOD	1.32 ng/ml	0.90 ng/ml
Resolution	3.52	
K	4.17	2.83

8. Analysis of formulation:

The percentage of drug in formulation, mean and relative standard deviation were calculated. The result of analysis showed that the amount of drug present in the formulation is in good correlation with the label claim of the formulation.

Table17: analysis of formulation

Formulation	Labelled amount (mg)		Amount Found (mg)		Percentage assay (%)		%R.S.D*	
Tablet	TENE	GLI	TENE	GLI	TENE	GLI	TENE	GLI
	20	1	19.8	0.98	98.80	99.68	0.23	0.26

*mean of five observations

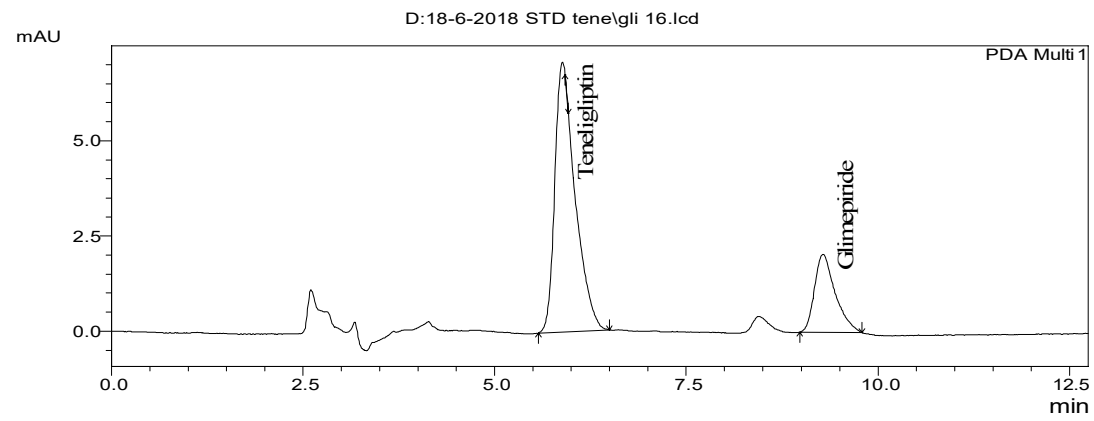

Fig. 8: Chromatogram for TENE 5 µg/ml and GLI 0.25 µg/ml (standard)

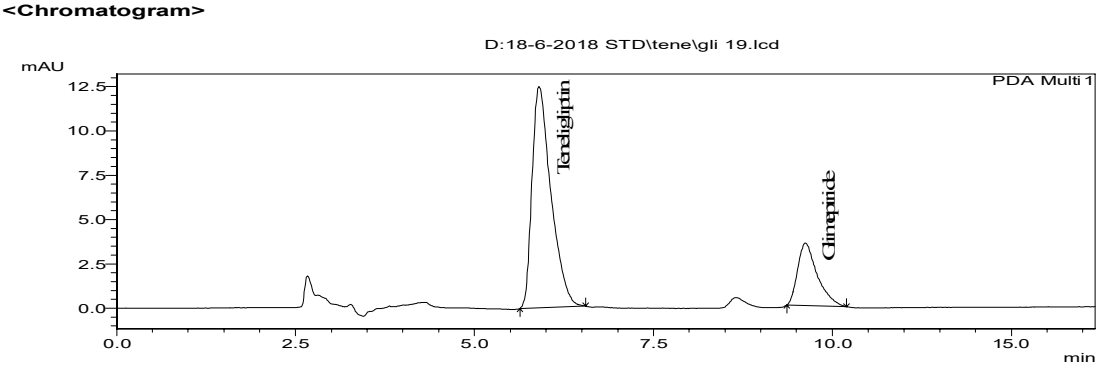

Fig.9: Chromatogram for TENE 10 µg/ml and GLI 0.50 µg/ml (standard)

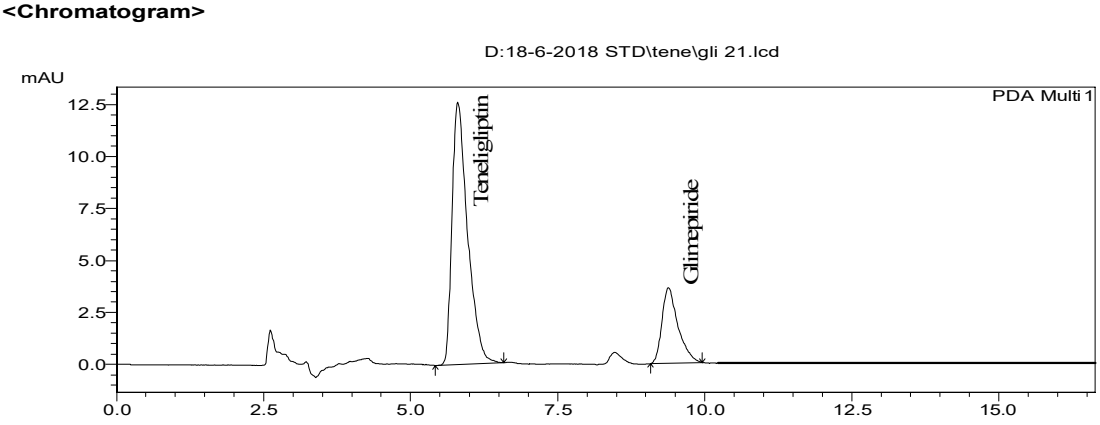

Fig.10: Chromatogram for TENE15 µg/ml and GLI 0.75 µg/ml (standard)

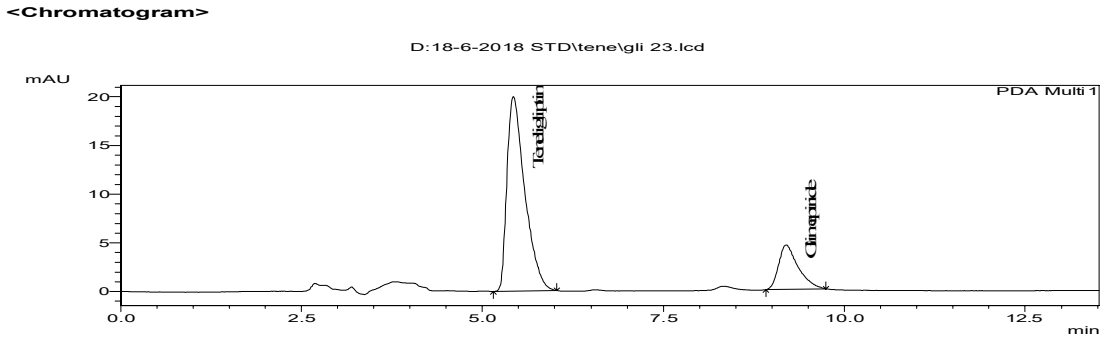

Fig.11: Chromatogram for TENE 20 µg/ml and GLI 1.00 µg/ml (standard)

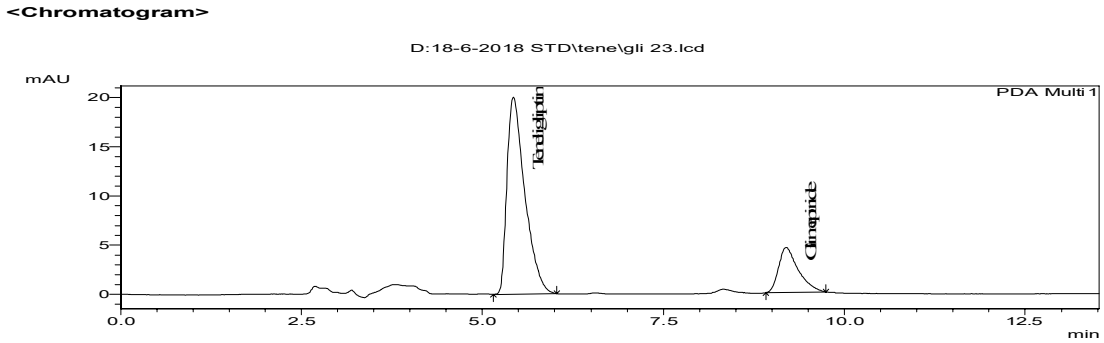

Fig.12: Chromatogram for TENE 25 µg/ml and GLI 1.25 µg/ml (standard)

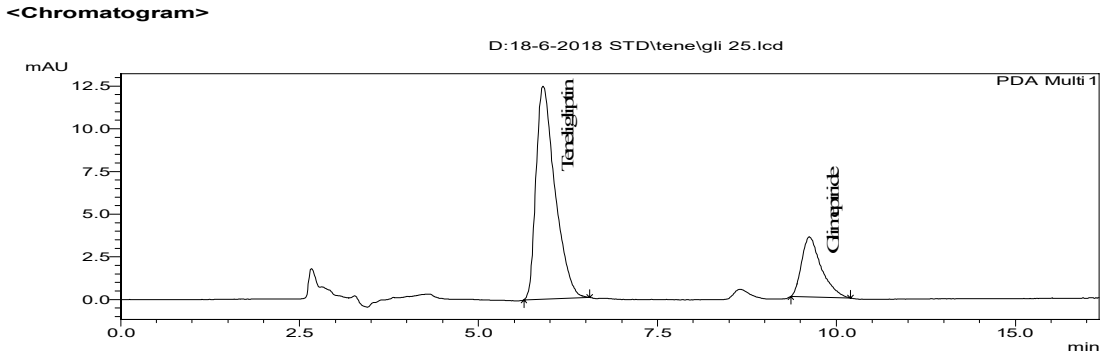

Fig.13: Chromatogram of TENE and GLI 15 µg/ml and 0.75 µg/ml (sample solution)

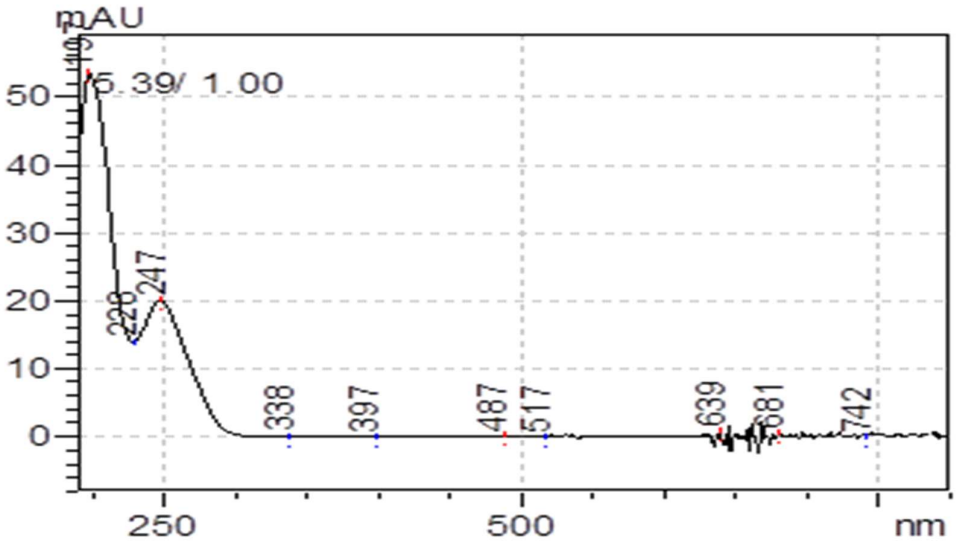

Fig.14: UV spectrum of Teneligliptin hydrobromide hydrate

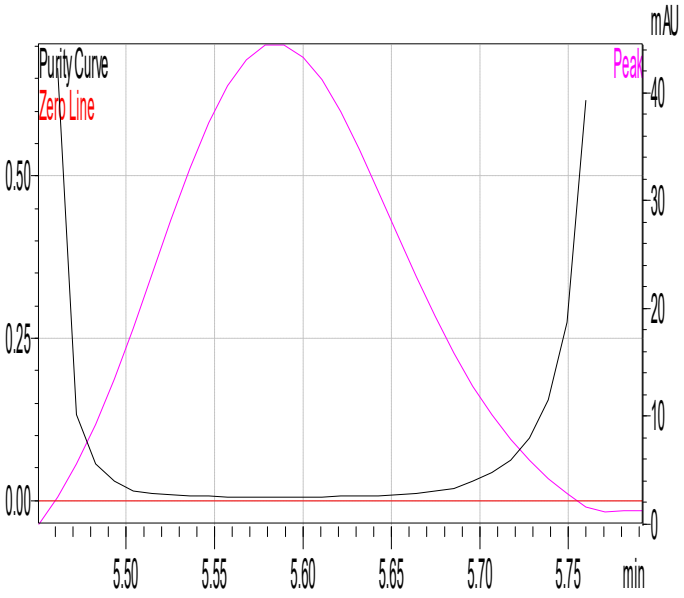

Fig.15: Purity curve of Teneligliptin hydrobromide hydrate

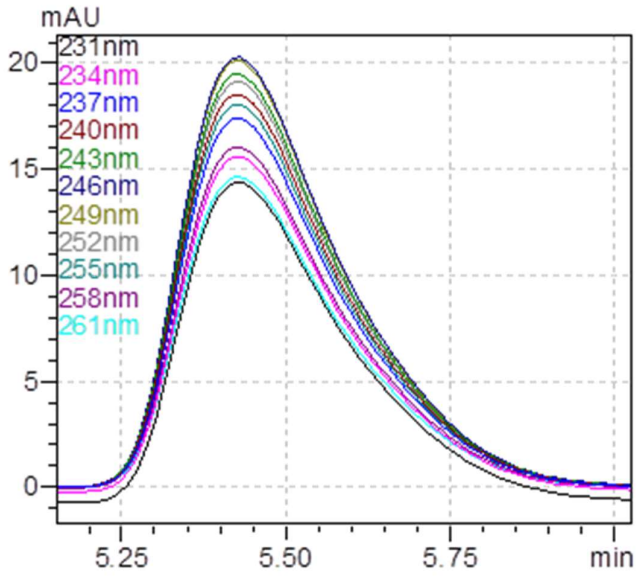

Fig.16: Peak profile of Teneligliptin hydrobromide hydrate

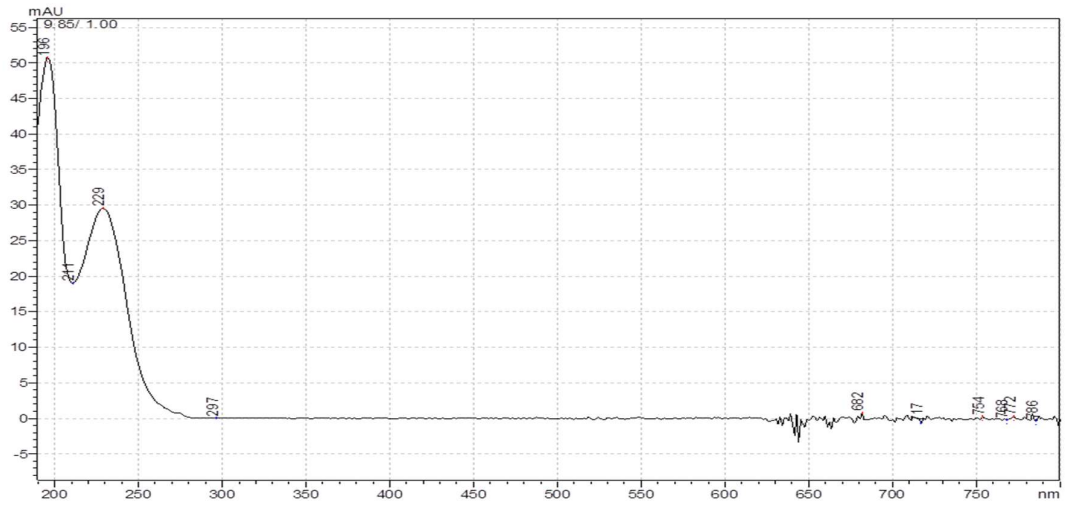

Fig.17: UV spectrum Glimepiride

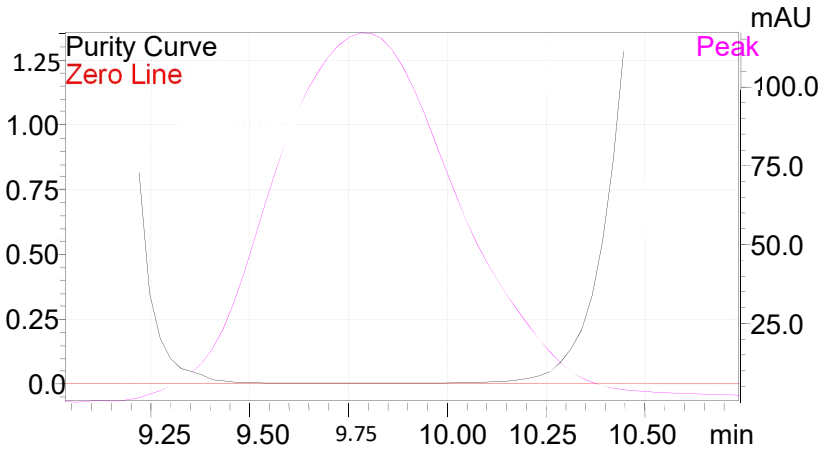

Fig.18: Peak purity of Glimepiride

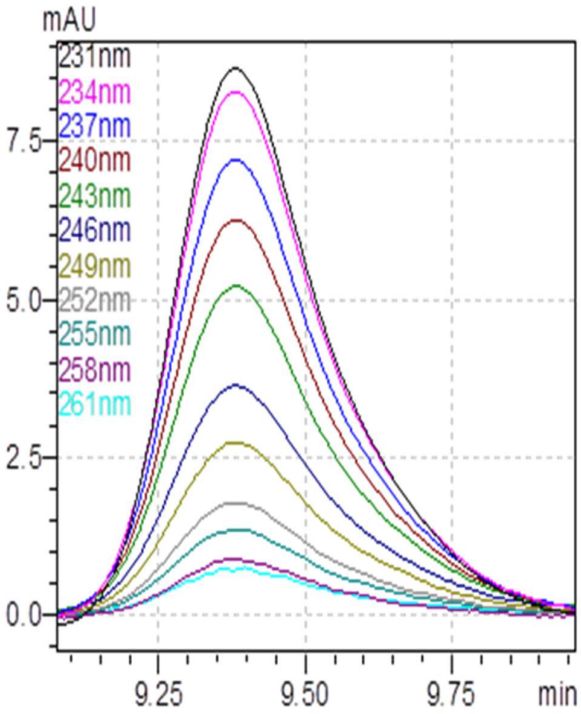

Fig.19: Peak profile of Glimepiride

HPTLC

A HPTLC method was developed for the simultaneous estimation of Teneligliptin hydrobromide hydrate and Glimepiride in tablet dosage forms, which can be conveniently employed for routine quality control in pharmaceutical dosage forms.

1. Linearity:

Teneligliptin hydrobromide hydrate and Glimepiride were found to be linear in the range of 2000 to 20000 (ng/spot) and 100 to 1000 (ng/spot) respectively.

The correlation coefficient of Teneligliptin hydrobromide hydrate and Glimepiride were found to be 0.9967 and 0.9938. The linearity range of Teneligliptin hydrobromide hydrate and Glimepiride were shown in **Table 18** respectively.

The calibration curves were plotted as peak area Vs concentration of the standard solutions **(Fig.20 and 21).**

The calibration curves graph shows that linear response was obtained over the range of concentrations used in the assay procedures. The range demonstrates that the method is linear outside the limits of expected use.

Table 18: Linearity range of Teneligliptin hydrobromide hydrate and glimepiride

Concentration (ng/spot)	TENE		Concentration (ng/spot)	GLI	
	R_f value	Peak area*		R_f value	Peak area*
2000	0.58	3020.3	100	0.46	2280.2
4000	0.58	5347.2	200	0.46	4203.5
8000	0.60	9932.4	400	0.46	7156.5
16000	0.59	17696.5	800	0.47	12402.3
20000	0.60	21686.2	1000	0.48	15382.1

*mean of five observations

Acceptance criteria

The results complied with an acceptance criteria since coefficient of correlation was found to be with in the limit i.e., NLT 0.99.

Calibration curve of teneligliptin

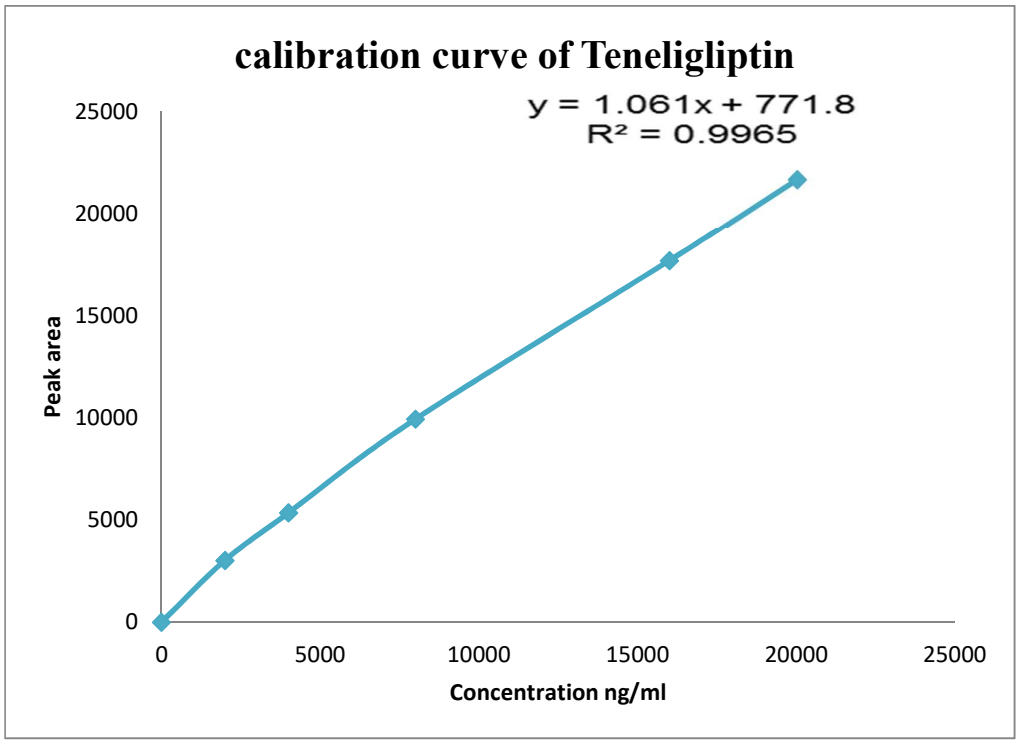

Fig.20: Calibration curve of Teneligliptin hydrobromide hydrate

Calibration curve of glimepiride

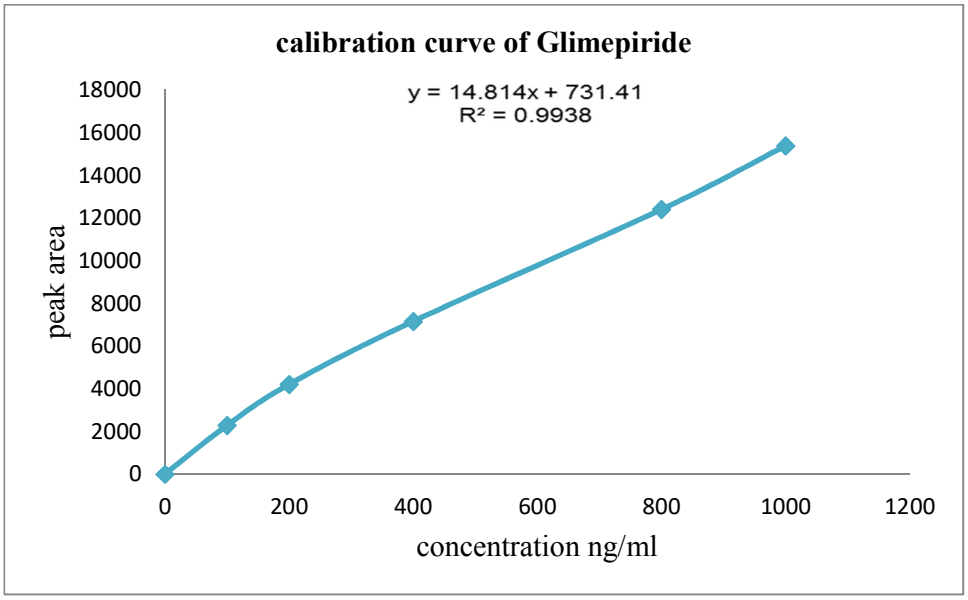

Fig.21: Calibration curve of Glimepiride

2. Accuracy (Recovery studies):

The accuracy of the method was determined by recovery experiments. A known quantity of the pure drug was added to the pre-analyzed sample formulations at 50%, 100% and 150% levels. The recovery studies were carried out 6 times of each level and the percentage recovery and percentage relative standard deviation were calculated and given in **Table 19.** The percentage recovery of Teneligliptin hydrobromide hydrate and Glimepiride found to be in the range of 99.56 to 99.87% and 99.64 to 99.92% respectively.

Table 19: Accuracy (Recovery studies)

Drug	Amount present	Spike Level (%)	Amount of drug added (ng/spot)	Amount of drug recovered (ng/spot)	Percentage Recovery	%RSD*
TENE	8000	50	4000	3989.7	99.65	0.75
		100	8000	7998.9	99.56	0.64
		150	12000	11988.7	99.87	0.88
GLI	400	50	200	199.7	99.78	1.113
		100	400	398.5	99.64	1.012
		150	600	598.7	99.92	1.203

*mean of five observations

From the data obtained, it was observed that the recoveries of standard drugs were found to be accurate and within the specified limits.

3. Precision:

The precision of the method was determined by studying reproducibility and repeatability. The area of drug peaks and percentage relative standard deviation of intraday and inter day were calculated and presented in **Table 20**. The results revealed that the developed method was found to be reproducible in nature.

Table 20: Intra-day and inter-day precision of the developed method

Drug	Concentration (ng/spot)	Intraday			Interday		
		Peak area*	SD	%RSD*	Peak area*	SD	%RSD*
TENE	4000	5382.2	48.12	0.89	5392.5	51.89	0.96
	8000	9982.5	102.36	1.02	9986.3	105.05	1.05
	16000	17637.5	294.54	1.47	17983.6	256.80	1.63
GLI	200	4218.2	46.01	1.09	4209.3	60.77	1.44
	400	7128.5	83.58	1.17	7978.5	95.96	1.22
	800	12853.5	212.10	1.54	12448.4	169.16	1.63

*mean of five observations

Acceptance criteria

The results complied with an acceptance criteria since the percentage relative standard deviation of peak areas of TENE and GLI were found to be with in the limit ie, NMT 2%.

Table 21: Repeatability

Conc. of TENE (ng/spot)	Peak Area*	% RSD*	Conc. of GLI (ng/spot)	Peak Area*	% RSD*
8000	9987.6	0.98	400	7198.4	1.41

* mean of five observations

Acceptance criteria

The results complied with an acceptance criteria since the percentage relative standard deviation was found to be within limit ie, NMT 2%.

4. LOD and LOQ:

Table 22: LOD and LOQ

Parameter	TENE (ng/spot)	GLI (ng/spot)
LOD	24.0	6.24
LOQ	72.74	4.93

* mean of five observations

5. Ruggedness:

Table 23: Ruggedness

Drug	Concentration (ng/spot)	Mean Peak area*	% R.S.D*
Day I, Analyst I			
TENE	8000	9958.6	1.12
GLI	400	7169.9	1.04
Day II, Analyst II			
TENE	8000	9989.7	1.23
GLI	400	7235.7	1.18

*mean of five values.

The sample was analyzed by a different chemist and same instruments on a different day. The method is rugged because the percentage relative standard deviation was found to be with in the limit i.e., NMT 2%.

6. Robustness:

The Robustness studies were performed for the standard solutions and presented in **Table 24.** This method was found to be robust because the percentage recovery were within the limit ie, ± 2%.

Table 24: Robustness studies

Parameter	Modification	Percentage Recovery (%)	
		TENE	GLI
Mobile Phase Ratio	1.5:2:1.5	98.43	99.56
	1:1.5:2.5	98.32	98.56
Development Distance	9 mm	98.56	98.92
Detection Wavelength(nm)	244 nm	98.96	98.82
Slit Dimension	5.00 x .30m micro	98.65	98.76

7. Analysis of formulation:

Table 25: Assay Teneligliptin hydrobromide hydrate and Glimepiride in tablet dosage form

Formulation	Labelled amount (mg)		Amount Found (mg)		Percentage assay (%)		%R.S.D*	
	TENE	GLI	TENE	GLI	TENE	GLI	TENE	GLI
	20	1	19.98	0.99	99.28	99.64	1.02	1.17

*mean of five observations

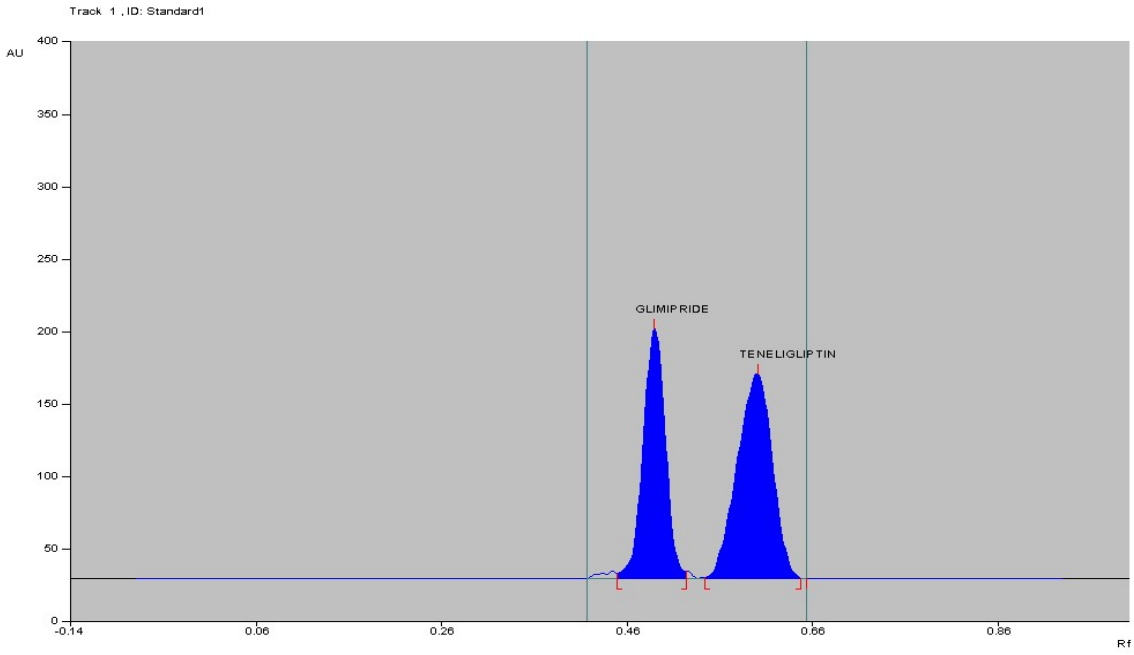

Fig. 22: Chromatogram of standard (100 ng/spot of GLI and 2000 ng/spot TENE)

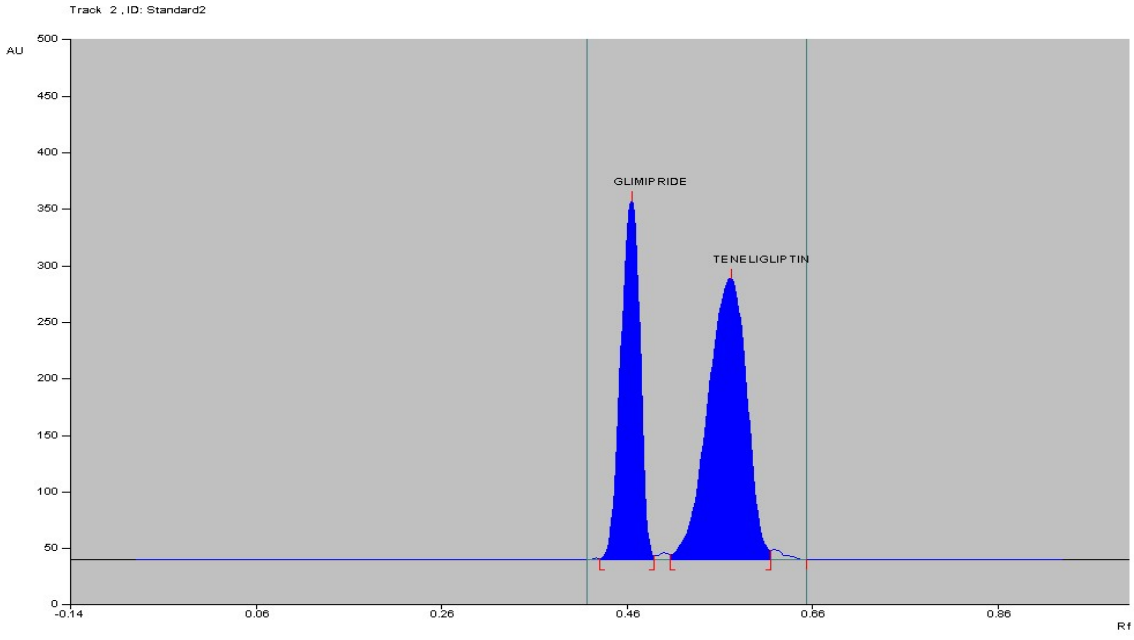

Fig. 23: Chromatogram of standard (200 ng/spot of GLI and 4000 ng/spot TENE)

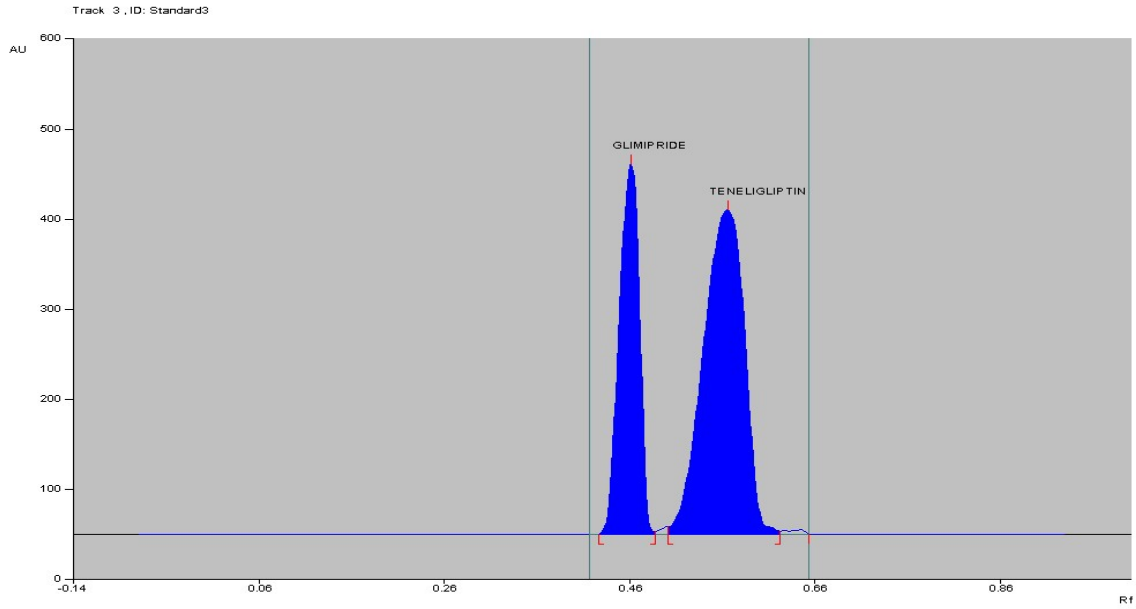

Fig. 24: Chromatogram of standard (400 ng/spot of GLI and 8000 ng/spot TENE)

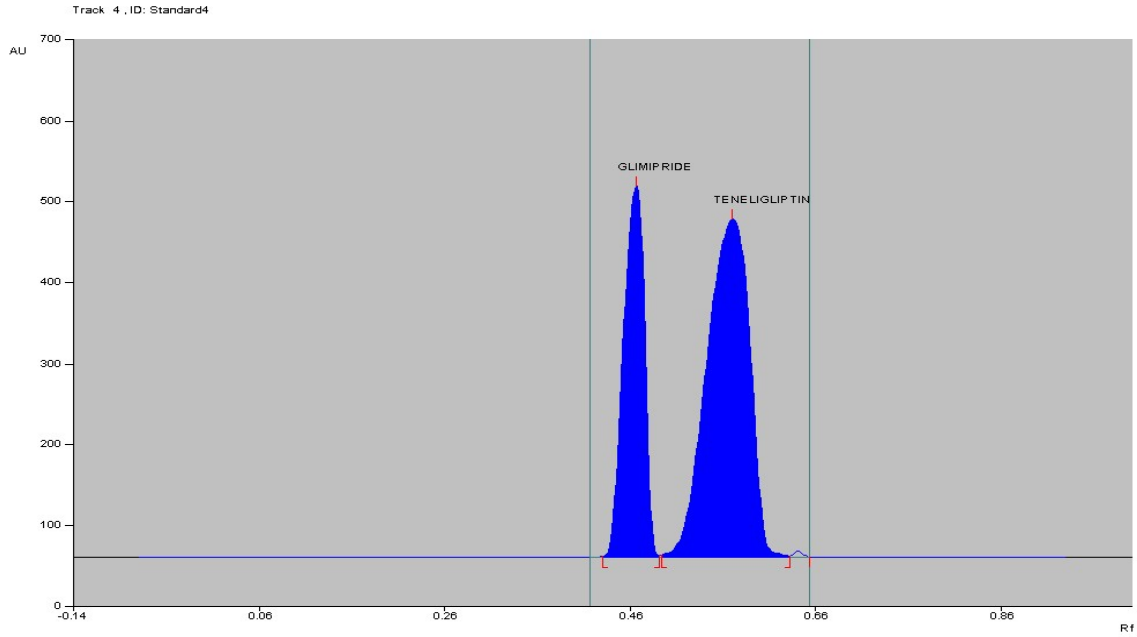

Fig. 25: Chromatogram of standard (800 ng/spot of GLI and 16000 ng/spot TENE)

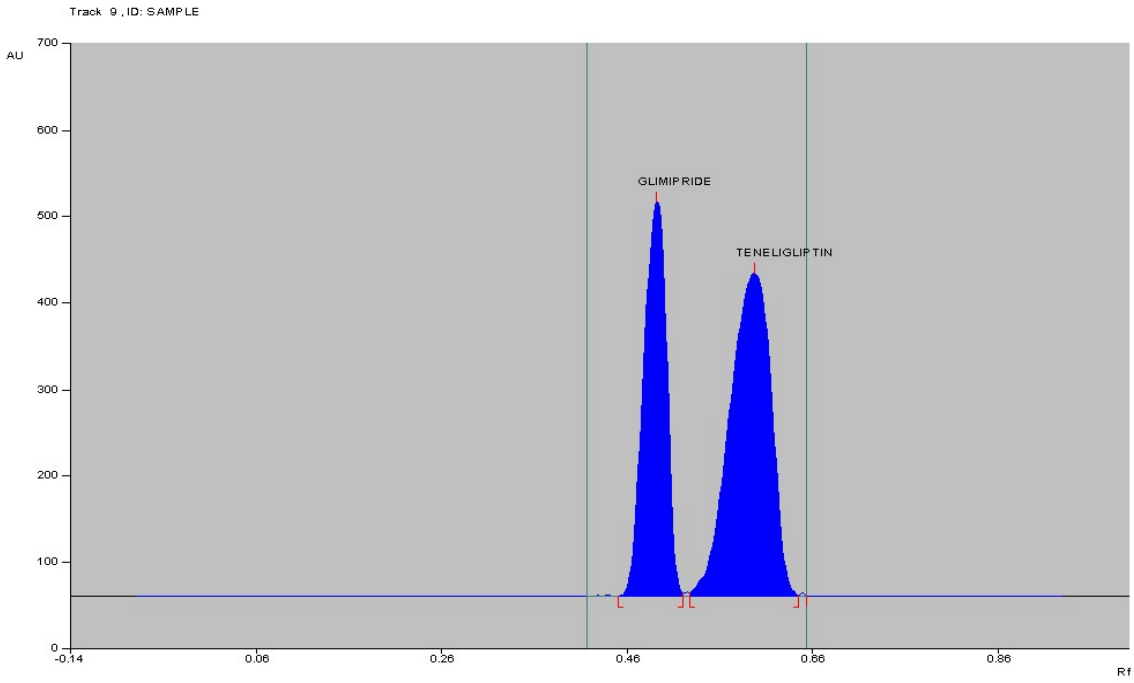

Fig. 26: Chromatogram of standard (1000ng/spotof GLI and 20000 ng/spot TENE)

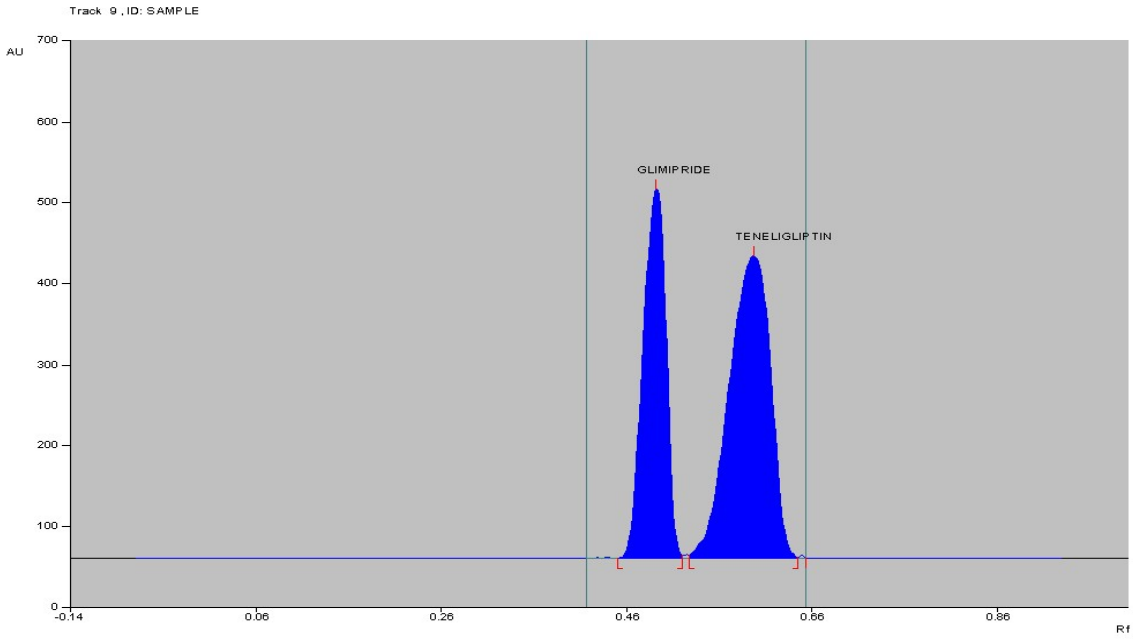

Fig. 27: Chromatogram of sample(8000 ng/spot of TENE and 400 ng/spot GLI)

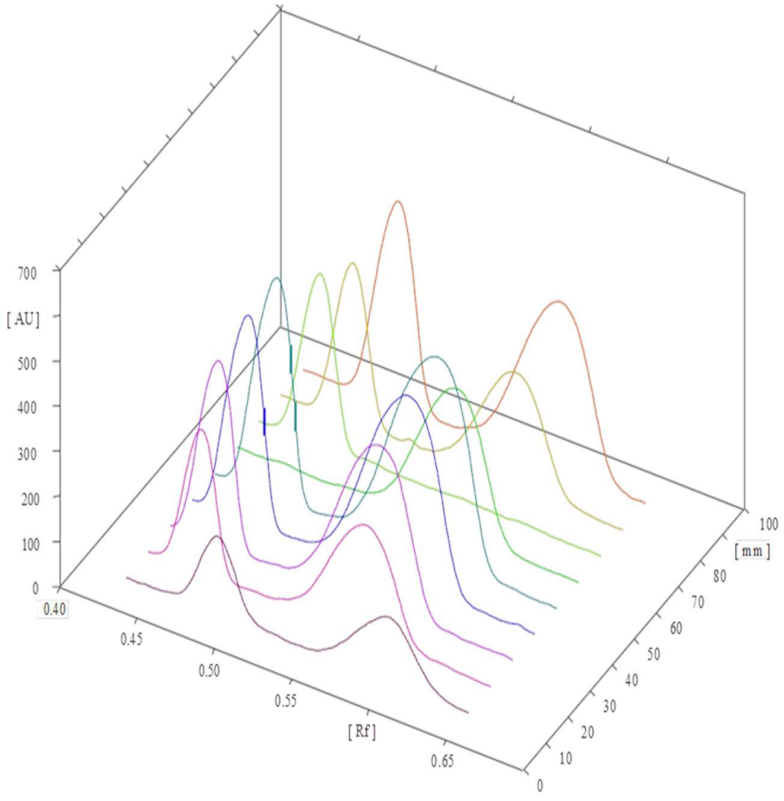

Fig. 28: Over all 3D diagram for TENE and GLI (standard and sample)

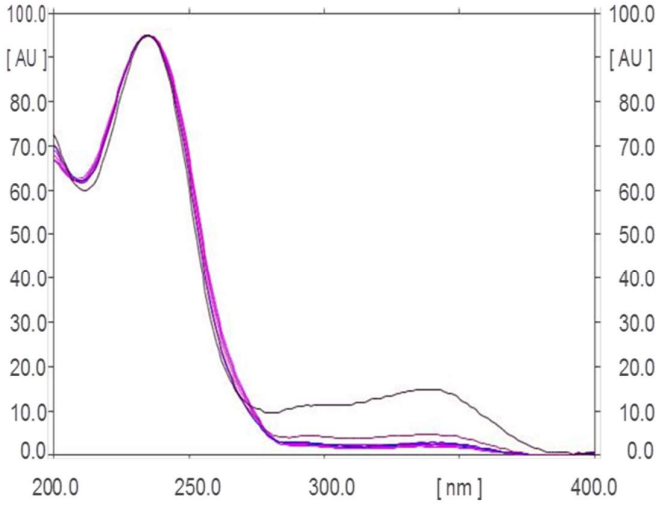

Fig. 29: Overlay spectrum of Glimepiride

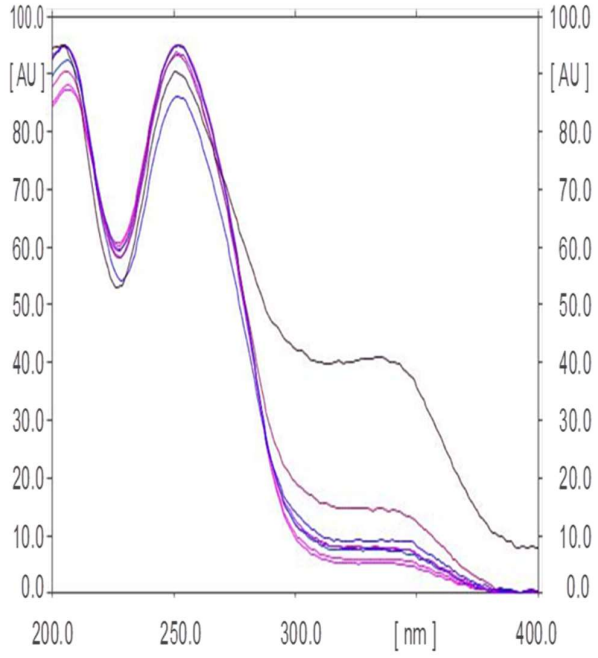

Fig. 30: Overlay spectrum of Teneligliptin

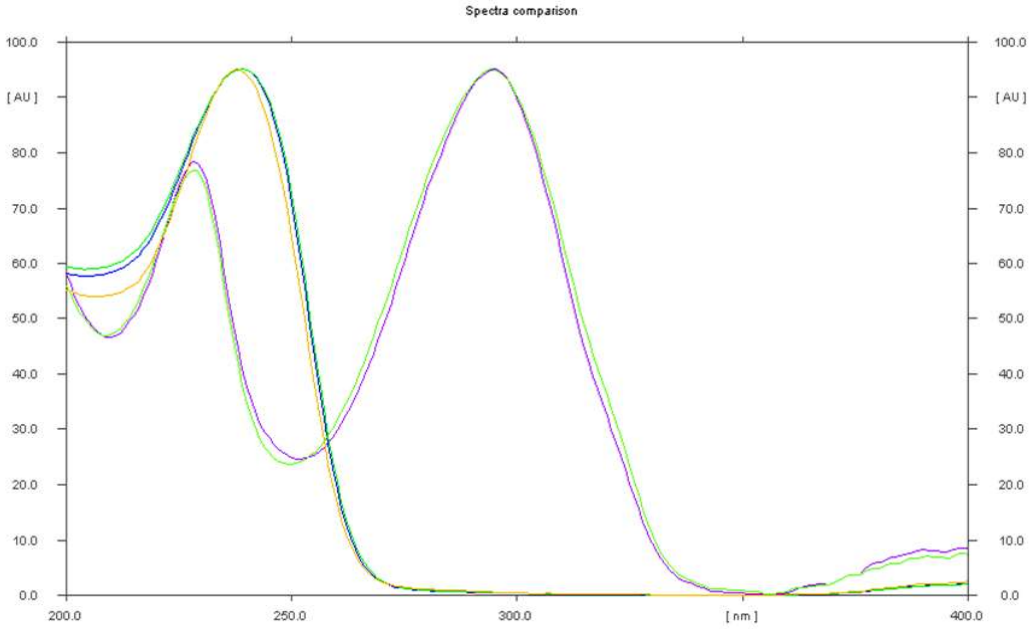

Fig 31: Overlay spectrum of standard (TENE and GLI)

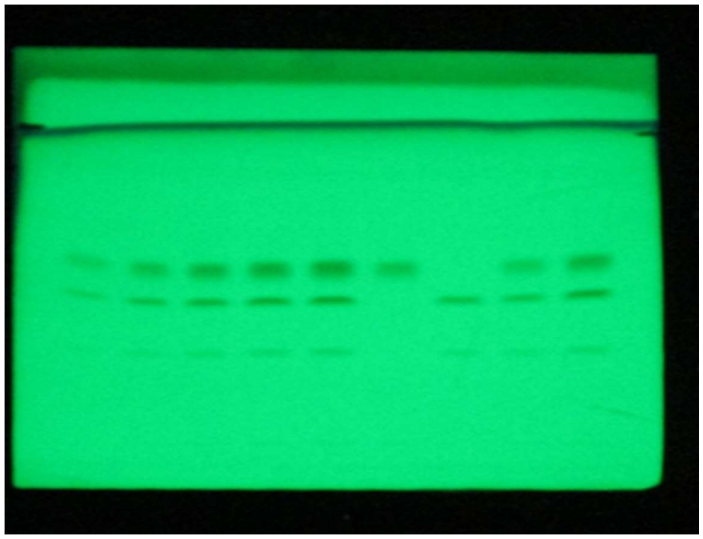

Fig. 32: Chromatogram of TENE and GLI (Standard and sample)

8. SUMMARY

The method was designed to validate RP-HPLC and HPTLC method for the determination of Teneligliptin hydrobromide hydrate and Glimepiride in bulk drug and formulations. The developed method was validated by using the following parameters like linearity, precision, accuracy, LOD, LOQ, system suitability, specificity, ruggedness and robustness. All the parameters were found to be satisfactory.

HPLC METHOD:

- A rapid, sensitive RP-HPLC method for the simultaneous determination of Teneligliptin hydrobromide hydrate and Glimepiride was developed and validated.
- The method development was carried out by using a mobile phase consisting of 65:35 of Acetonitrile: Phosphate Buffer was selected at pH 3.0 (which was adjusted with orthophosphoric acid). The detection was carried out by using PDA detector at 246nm. The column was phenomenex C18 (250 X 4.6mm, 5µ). The flow rate was selected as 1ml/min.
- The retention time of Teneligliptin hydrobromide hydrate and Glimepiride was found to be 5.8min and 9.41min respectively.
- The linearity range of Teneligliptin hydrobromide hydrate 5-25 µg/ml and Glimepiride 0.25-1.25 µg/ml was found to be obeying linearity with the correlation coefficient of 0.99 respectively.
- The proposed and validated method was successfully applied to determine Teneligliptin hydrobromide hydrate and Glimepiride in their combined dosage form. The results obtained for Teneligliptin hydrobromide hydrate and Glimepiride were comparable with the corresponding labeled amounts.
- The tailing factor of is Teneligliptin hydrobromide hydrate and Glimepiride 0.98 and 1.02 and the number of theoretical plates were found to be 5069 and 5909 respectively, indicating the efficiency of column and these parameters represent the specificity of the method.
- The recovery experiment was performed by the standard addition method. The percentage recovery of Teneligliptin hydrobromide hydrate and

Glimepiride was found to be 98.83 to 99.83% and 99.60 to 99.90% respectively. The results indicate that proposed method is highly accurate.

- The RSD values of Teneligliptin hydrobromide hydrate and Glimepiride interday (0.35-0.36% and 0.23-0.25%) and intraday (0.39% and 0.25%), reveal that the proposed method is precise.
- LOD and LOQ values for Teneligliptin hydrobromide hydrate (1.32 µg/ml and 3.56 µg/ml) and (0.90 µg/ml and 2.73 µg/ml) showed that the method is sensitive for the determination of Teneligliptin hydrobromide hydrate and Glimepiride.
- There is no other co eluting peak with the main peaks, hence the RP-HPLC method is specific for the estimation of Teneligliptin hydrobromide hydrate and Glimepiride.
- The validated method was found to be robust.
- The ruggedness of the method demonstrated that different operational and environmental variables had only a minimal influence on the test results.

HPTLC METHOD:

- A HPTLC method has been developed and validated for the simultaneous estimation of Teneligliptin hydrobromide hydrate and Glimepiride in pure and pharmaceutical dosage form.
- Good separation of analytes were achieved by using Toluene: Methanol: Triethylamine (1:3:1 v/v/v) as a mobile phase on precoated silica gel 60F 254 plates.
- The Rf values for Glimepiride and Teneligliptin hydrobromide hydrate were found to be 0.48 and 0.60 respectively. The linearity range of Glimepiride (100 – 1000 ng/spot) and Teneligliptin hydrobromide hydrate (2000 – 20000 ng/spot) was found obey linearity with a correlation coefficient of 0.9935 and 0.9968 respectively.
- The proposed validated method was successfully applied to determine Teneligliptin hydrobromide hydrate and Glimepiride in combined dosage form. The results obtained for Teneligliptin hydrobromide hydrate and Glimepiride was comparable with the corresponding labeled amounts.

- The percentage recovery of Teneligliptin hydrobromide hydrate and Glimepiride was found to be 99.56 to 99.87% and 99.64 to 99.92%, which indicate that proposed method is highly accurate.
- The RSD values of Teneligliptin hydrobromide hydrate and Glimepiride for interday (0.96-1.63% and 1.22-1.63%) and intraday (0.89-1.47% and 1.09-1.54%), reveal that the proposed method is precise.
- LOD and LOQ for Teneligliptin hydrobromide hydrate was 24.0 and 72.74 (ng/spot) and for Glimepiride was 6.24 and 4.93 ng/spot respectively.
- There is no other co eluting peak with the main peaks, hence the HPTLC method is specific for the estimation of Teneligliptin hydrobromide hydrate and Glimepiride.
- The validated method was found to be robust. The ruggedness of the method demonstrated that different operational and environmental variables had only a minimal influence on the test results.

9. CONCLUSION

- The novel RP-HPLC and HPTLC methods were validated by determining system suitability, specificity, precision, linearity, accuracy, stability, LOD, LOQ, ruggedness and robustness parameters and found to be satisfactory both in RP-HPLC and HPTLC method.
- The standard preparations were done in the range of 5-25 µg/ml for TENE and 0.25-1.25 µg/ml for GLI. The assay concentration were linear (correlation coefficient, R^2=0.9983 and 0.9967, n=5) in the developed RP-HPLC method.
- There was no other co eluting peak with TENE and GLI peaks and hence both RP-HPLC and HPTLC methods are specific for the estimation of both TENE and GLI in the presence of other excipients.
- In HPLC, the flow rate is reduced (1ml/min) as compared with previously reported methods in literatures.
- The developed method is eco-friendly as the mobile phase used is biodegradable.
- It is also economic since the solvent used is of lower cost when compared with the other literatures already reported.
- In HPTLC a newer method has been developed.
- The standard preparations in the range of 2000 (ng/spot) to 20000 (ng/spot) for TENE and 100 (ng/spot) to 1000 (ng/spot) for GLI. The assay standard concentration were linear for both TENE and GLI (correlation coefficient, R^2=0.9966 and 0.9938, n=5) in developed HPTLC method.
- The ruggedness of both the RP-HPLC and HPTLC method demonstrated that different operational and environmental variables had very little influence with in the limit on the test results.
- The method was completely validated showing satisfactory data for all the method validation parameters that were tested.
- It was concluded that, the developed method offered several advantages such as, rapid, cost effective, simple mobile phase, comparative short run time made it specific and reliable and it is in good agreement with the label claim of the drug. The additives present in the pharmaceutical formulation of the assayed sample did not interfere with determination of TENE and GLI.
- Both RP-HPLC and HPTLC methods can be used for routine analysis of TENE and GLI in their combined dosage form without any interference of excipients.

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11. ABSTRACT

A novel RP-HPLC and HPTLC method were developed and validated for the simultaneous estimation of Teneligliptin hydrobromide hydrate and Glimepiride.RP-HPLC method was developed in reverse phase mode using acetonitrile and phosphate buffer (pH 3 adjusted with orthophosphoric acid) as mobile phase in the ratio of 65:35 (v/v) at a flow rate 1ml/min and. Quantitation was achieved with ultraviolet detection at 246nm and retention time was found to be 5.8 min and 9.41 min for Teneligliptin hydrobromide hydrate and Glimepiride respectively. The linearity was found to be in the range of 5 – 25 ng/ml and 0.25 – 1.25 ng/ml and recovery were found to be 98.80 and 99.68 % for Teneligliptin hydrobromide hydrate and Glimepiride respectively. The developed method is co-friendly and the peaks were more resolved when compared to the previous literatures with reduced flow rate. HPTLC method wasdeveloped on Merck TLC aluminium sheets of silica gel 60 F□□□ using Toluene: Methanol: Triethylamine (1: 3: 1 v/v/v) as mobile phase. Quantitation was achieved with ultraviolet detection a 246 nm and Rf value was found to be 0.48 and 0.60 for Glimepiride and Teneligliptin hydrobromide hydrate respectively. The linearity was found to be in the range of 2000 – 20000 ng/spot and 100 – 1000 ng/spot and mean recovery was found to be 99.64 and 99.28 % of Glimepiride and Teneligliptin hydrobromide hydrate respectively. Both HPLC and HPTLC were found to be specific since there was no co-eluting peak with drug